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(71) Applicant *(for all designated States except US)*: **BIONOMICS LIMITED** [AU/AU]; 31 Dalgleish Street, Thebarton, South Australia 5031 (AU).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GAMBLE, Jennifer, Ruth** [AU/AU]; 8 Branch Road, Stirling, South Australia 5152 (AU). **HAHN, Christopher, Norman** [AU/AU]; 21 Wedgwood Road, Modbury Heights, South Australia 5092 (AU). **VADAS, Mathew, Alexander** [AU/AU]; 8 Branch Road, Stirling, South Australia 5152 (AU).

(74) Agent: **GRIFFITH HACK**; 10/167 Eagle Street, GPO Box 3125, Brisbane, Queensland 4001 (AU).

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(54) Title: DNA SEQUENCES FOR HUMAN ANGIOGENESIS GENES

(57) Abstract: An isolated nucleic acid molecule comprising the sequence set forth in one of SEQ ID Numbers: 1 to 20.

DNA SEQUENCES FOR HUMAN ANGIOGENESIS GENES

Technical Field

The present invention relates to novel nucleic acid sequences ("angiogenic genes") involved in the process of angiogenesis. Each of the angiogenic genes encode a polypeptide that has a role in angiogenesis. In view of the realisation that these genes play a role in angiogenesis, the invention is also concerned with the therapy of pathologies associated with angiogenesis, the screening of drugs for pro- or anti-angiogenic activity, the diagnosis and prognosis of pathologies associated with angiogenesis, and in some cases the use of the nucleic acid sequences to identify and obtain full-length angiogenesis-related genes.

Background Art

The formation of new blood vessels from pre-existing vessels, a process termed angiogenesis, is essential for normal growth. Important angiogenic processes include those taking place in embryogenesis, renewal of the endometrium, formation and growth of the corpus luteum of pregnancy, wound healing and in the restoration of tissue structure and function after injury.

The formation of new capillaries requires a co-ordinated series of events mediated through the expression of multiple genes which may have either pro- or anti-angiogenic activities. The process begins with an angiogenic stimulus to existing vasculature, usually mediated by growth factors such as vascular endothelial growth factor or basic fibroblast growth factor. This is followed by degradation of the extracellular matrix, cell adhesion changes (and disruption), an increase in cell permeability, proliferation of endothelial cells (ECs) and migration of ECs towards the site of blood vessel formation. Subsequent processes include capillary tube or lumen formation, stabilisation and differentiation by the migrating ECs.

In the (normal) healthy adult, angiogenesis is virtually arrested and occurs only when needed. However, a number of pathological situations are characterised by enhanced, uncontrolled angiogenesis. These conditions include cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis and cardiovascular diseases such as atherosclerosis. In other pathologies such as ischaemic limb disease or in coronary artery disease, growing new vessels through the promotion of an expanding vasculature would be of benefit.

A number of *in vitro* assays have been established which are thought to mimic angiogenesis and these have provided important tools to examine the mechanisms by which the angiogenic process takes place and the genes most likely to be involved.

Lumen formation is a key step in angiogenesis. The presence of vacuoles within ECs undergoing angiogenesis have been reported and their involvement in lumen formation has been postulated (Folkman and Haudenschild, 1980; Gamble et al., 1993). The general mechanism of lumen formation suggested by Folkman and Haudenschild (1980), has been that vacuoles form within the cytoplasm of a number of aligned ECs which are later converted to a tube. The union of adjacent tubes results in the formation of a continuous unicellular capillary lumen. However, little is known about the changes in cell morphology leading to lumen formation or the signals required for ECs to construct this feature.

An *in vitro* model of angiogenesis has been created from human umbilical vein ECs plated onto a 3 dimensional collagen matrix (Gamble et al., 1993). In the presence of phorbol myristate acetate (PMA) these cells form capillary tubes within 24 hours. With the addition of anti-integrin antibodies, the usually unicellular tubes (thought to reflect an immature, poorly differentiated phenotype) are converted to form a multicellular lumen through the inhibition of cell-matrix interactions and promotion of

cell-cell interactions. This model has subsequently allowed the investigation of the morphological events which occur in lumen formation.

For the treatment of diseases associated with angiogenesis, understanding the molecular genetic mechanisms of the process is of paramount importance. The use of the *in vitro* model described above (Gamble et al., 1993), a model that reflects the critical events that occur during angiogenesis *in vivo* in a time dependant and broadly synchronous manner, has provided a tool for the identification of the key genes involved.

A number of genes have been identified from this model to be differentially expressed during the angiogenesis process. Functional analysis of a subset of these angiogenic genes and their effect on endothelial cell function and proliferation is described in detail below.

The isolation of these angiogenic genes has provided novel targets for the treatment of angiogenesis-related disorders.

Disclosure of the Invention

The present invention provides isolated nucleic acid molecules, which have been shown to be regulated in their expression during angiogenesis (see Tables 1 and 2).

In a first aspect of the present invention there is provided an isolated nucleic acid molecule as defined by SEQ ID Numbers: 1 to 20 and laid out in Table 1.

Following the realisation that these molecules, and those listed in Table 2, are regulated in their expression during angiogenesis, the invention provides isolated nucleic acid molecules as defined by SEQ ID Numbers: 1 to 114, and laid out in Tables 1 and 2, or fragments thereof, that play a role in an angiogenic process. Such a process may include, but is not restricted to, embryogenesis, menstrual cycle, wound repair, tumour angiogenesis and exercise induced muscle hypertrophy.

In addition, the present invention provides isolated nucleic acid molecules as defined by SEQ ID Numbers: 1 to 114, and laid out in Tables 1 and 2 (hereinafter referred to as "angiogenic genes", "angiogenic nucleic acid molecules" or "angiogenic polypeptides" for the sake of convenience), or fragments thereof, that play a role in diseases associated with the angiogenic process. Diseases may include, but are not restricted to, cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis, cardiovascular diseases such as atherosclerosis, ischaemic limb disease and coronary artery disease.

The invention also encompasses an isolated nucleic acid molecule that is at least 70% identical to any one of the angiogenic genes of the invention and which plays a role in the angiogenic process.

Such variants will have preferably at least about 85%, and most preferably at least about 95% sequence identity to the angiogenic genes. Any one of the polynucleotide variants described above can encode an amino acid sequence, which contains at least one functional or structural characteristic of the relevant angiogenic gene of the invention.

Sequence identity is typically calculated using the BLAST algorithm, described in Altschul *et al* (1997) with the BLOSUM62 default matrix.

The invention also encompasses an isolated nucleic acid molecule which hybridises under stringent conditions with any one of the angiogenic genes of the invention and which plays a role in an angiogenic process.

Hybridisation with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, may be used to identify nucleic acid sequences which encode the relevant angiogenic gene. The specificity of the probe, whether it is made from a highly specific region, e.g., the 5' regulatory region, or from a less specific region, e.g., a conserved motif, and the stringency of the hybridisation or amplification will

determine whether the probe identifies only naturally occurring sequences encoding the angiogenic gene, allelic variants, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably have at least 50% sequence identity to any of the angiogenic gene encoding sequences of the invention. The hybridisation probes of the subject invention may be DNA or RNA and may be derived from any one of the angiogenic gene sequences or from genomic sequences including promoters, enhancers, and introns of the angiogenic genes.

Means for producing specific hybridisation probes for DNAs encoding any one of the angiogenic genes include the cloning of polynucleotide sequences encoding the relevant angiogenic gene or its derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, and are commercially available. Hybridisation probes may be labelled by radionuclides such as ^{32}P or ^{35}S, or by enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, or other methods known in the art.

Under stringent conditions, hybridisation with ^{32}P labelled probes will most preferably occur at 42°C in 750 mM NaCl, 75 mM trisodium citrate, 2% SDS, 50% formamide, 1X Denhart's, 10% (w/v) dextran sulphate and 100 µg/ml denatured salmon sperm DNA. Useful variations on these conditions will be readily apparent to those skilled in the art. The washing steps which follow hybridisation most preferably occur at 65°C in 15 mM NaCl, 1.5 mM trisodium citrate, and 1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art.

The nucleotide sequences of the present invention can be engineered using methods accepted in the art so as to alter angiogenic gene-encoding sequences for a variety of purposes. These include, but are not limited to, modification of the cloning, processing, and/or expression

of the gene product. PCR reassembly of gene fragments and the use of synthetic oligonucleotides allow the engineering of angiogenic gene nucleotide sequences. For example, oligonucleotide-mediated site-directed mutagenesis can introduce mutations that create new restriction sites, alter glycosylation patterns and produce splice variants etc.

As a result of the degeneracy of the genetic code, a number of polynucleotide sequences encoding the angiogenic genes of the invention, some that may have minimal similarity to the polynucleotide sequences of any known and naturally occurring gene, may be produced. Thus, the invention includes each and every possible variation of polynucleotide sequence that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the polynucleotide sequence of the naturally occurring angiogenic gene, and all such variations are to be considered as being specifically disclosed.

The polynucleotides of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified, as will be appreciated by those skilled in the art. Such modifications include labels, methylation, intercalators, alkylators and modified linkages. In some instances it may be advantageous to produce nucleotide sequences encoding an angiogenic gene or its derivatives possessing a substantially different codon usage than that of the naturally occurring gene. For example, codons may be selected to increase the rate of expression of the peptide in a particular prokaryotic or eukaryotic host corresponding with the frequency that particular codons are utilized by the host. Other reasons to alter the nucleotide sequence encoding an angiogenic gene or its derivatives without altering the encoded amino acid sequence include the production of RNA transcripts

having more desirable properties, such as a greater half-life, than transcripts produced from the naturally occurring sequence.

The invention also encompasses production of the nucleic acid molecules of the invention, entirely by synthetic chemistry. Synthetic sequences may be inserted into expression vectors and cell systems that contain the necessary elements for transcriptional and translational control of the inserted coding sequence in a suitable host. These elements may include regulatory sequences, promoters, 5′ and 3′ untranslated regions and specific initiation signals (such as an ATG initiation codon and Kozak consensus sequence) which allow more efficient translation of sequences encoding the angiogenic genes. In cases where the complete coding sequence including its initiation codon and upstream regulatory sequences are inserted into the appropriate expression vector, additional control signals may not be needed. However, in cases where only coding sequence, or a fragment thereof, is inserted, exogenous translational control signals as described above should be provided by the vector. Such signals may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate for the particular host cell system used (Scharf et al., 1994).

Nucleic acid molecules that are complements of the sequences described herein may also be prepared.

The present invention allows for the preparation of purified polypeptides or proteins. In order to do this, host cells may be transfected with a nucleic acid molecule as described above. Typically, said host cells are transfected with an expression vector comprising a nucleic acid molecule according to the invention. A variety of expression vector/host systems may be utilized to contain and express the sequences. These include, but are not limited to, microorganisms such as bacteria transformed with plasmid or cosmid DNA expression vectors; yeast

transformed with yeast expression vectors; insect cell systems infected with viral expression vectors (e.g., baculovirus); or mouse or other animal or human tissue cell systems. Mammalian cells can also be used to express a protein that is encoded by a specific angiogenic gene of the invention using various expression vectors including plasmid, cosmid and viral systems such as a vaccinia virus expression system. The invention is not limited by the host cell employed.

The polynucleotide sequences, or variants thereof, of the present invention can be stably expressed in cell lines to allow long term production of recombinant proteins in mammalian systems. Sequences encoding any one of the angiogenic genes of the invention can be transformed into cell lines using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. The selectable marker confers resistance to a selective agent, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be propagated using tissue culture techniques appropriate to the cell type.

The protein produced by a transformed cell may be secreted or retained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides which encode a protein may be designed to contain signal sequences which direct secretion of the protein through a prokaryotic or eukaryotic cell membrane.

In addition, a host cell strain may be chosen for its ability to modulate expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, glycosylation, phosphorylation, and acylation. Post-translational cleavage of a "prepro" form of the protein may also be

used to specify protein targeting, folding, and/or activity. Different host cells having specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO or HeLa cells), are available from the American Type Culture Collection (ATCC) and may be chosen to ensure the correct modification and processing of the foreign protein.

When large quantities of protein are needed such as for antibody production, vectors which direct high levels of expression may be used such as those containing the T5 or T7 inducible bacteriophage promoter. The present invention also includes the use of the expression systems described above in generating and isolating fusion proteins which contain important functional domains of the protein. These fusion proteins are used for binding, structural and functional studies as well as for the generation of appropriate antibodies.

In order to express and purify the protein as a fusion protein, the appropriate polynucleotide sequences of the present invention are inserted into a vector which contains a nucleotide sequence encoding another peptide (for example, glutathionine succinyl transferase). The fusion protein is expressed and recovered from prokaryotic or eukaryotic cells. The fusion protein can then be purified by affinity chromatography based upon the fusion vector sequence and the relevant protein can subsequently be obtained by enzymatic cleavage of the fusion protein.

Fragments of polypeptides of the present invention may also be produced by direct peptide synthesis using solid-phase techniques. Automated synthesis may be achieved by using the ABI 431A Peptide Synthesizer (Perkin-Elmer). Various fragments of polypeptide may be synthesized separately and then combined to produce the full length molecule.

In instances where the isolated nucleic acid molecules of the invention represent only partial gene sequence, these partial sequences can be used to obtain

the corresponding sequence of the full-length angiogenic gene. Therefore, the present invention further provides the use of a partial nucleic acid molecule of the invention comprising a nucleotide sequence defined by any one of SEQ ID Numbers: 70, 72 to 73, 78, 83 to 87, 89, 160 or 174 to identify and/or obtain full-length human genes involved in the angiogenic process. Full-length angiogenic genes may be cloned using the partial nucleotide sequences of the invention by methods known *per se* to those skilled in the art. For example, *in silico* analysis of sequence databases such as those hosted at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) can be searched in order to obtain overlapping nucleotide sequence. This provides a "walking" strategy towards obtaining the full-length gene sequence. Appropriate databases to search at this site include the expressed sequence tag (EST) database (database of GenBank, EMBL and DDBJ sequences from their EST divisions) or the non redundant (nr) database (contains all GenBank, EMBL, DDBJ and PDB sequences but does not include EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences). Typically searches are performed using the BLAST algorithm described in Altschul *et al* (1997) with the BLOSUM62 default matrix. In instances where *in silico* "walking" approaches fail to retrieve the complete gene sequence, additional strategies may be employed. These include the use of "restriction-site PCR" will allows the retrieval of unknown sequence adjacent to a portion of DNA whose sequence is known. In this technique universal primers are used to retrieve unknown sequence. Inverse PCR may also be used, in which primers based on the known sequence are designed to amplify adjacent unknown sequences. These upstream sequences may include promoters and regulatory elements. In addition, various other PCR-based techniques may be used, for example a kit available from Clontech (Palo Alto, California) allows for a walking PCR technique, the 5'RACE kit (Gibco-BRL) allows isolation of additional 5'

gene sequence, while additional 3′ sequence can be obtained using practised techniques (for eg see Gecz et al., 1997).

In a further aspect of the present invention there is provided an isolated polypeptide as defined by SEQ ID Numbers: 115 to 125 and laid out in Table 1.

The present invention also provides isolated polypeptides, which have been shown to be regulated in their expression during angiogenesis (see Tables 1 and 2).

More specifically, following the realisation that these polypeptides are regulated in their expression during angiogenesis, the invention provides isolated polypeptides as defined by SEQ ID Numbers: 115 to 216, and laid out in Tables 1 and 2, or fragments thereof, that play a role in an angiogenic process. Such a process may include, but is not restricted to, embryogenesis, menstrual cycle, wound repair, tumour angiogenesis and exercise induced muscle hypertrophy.

In addition, the present invention provides isolated polypeptides as defined by SEQ ID Numbers: 115 to 216, and laid out in Tables 1 and 2, or fragments thereof, that play a role in diseases associated with the angiogenic process. Diseases may include, but are not restricted to, cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis, cardiovascular diseases such as atherosclerosis, ischaemic limb disease and coronary artery disease.

The invention also encompasses an isolated polypeptide having at least 70%, preferably 85%, and more preferably 95% identity to any one of SEQ ID Numbers: 115 to 216, and which plays a role in an angiogenic process.

Sequence identity is typically calculated using the BLAST algorithm, described in Altschul *et al* (1997) with the BLOSUM62 default matrix.

In a further aspect of the invention there is provided a method of preparing a polypeptide as described above, comprising the steps of:

(1) culturing the host cells under conditions effective for production of the polypeptide; and

(2) harvesting the polypeptide.

According to still another aspect of the invention there is provided a polypeptide which is the product of the process described above.

Substantially purified protein or fragments thereof can then be used in further biochemical analyses to establish secondary and tertiary structure for example by x-ray crystallography of the protein or by nuclear magnetic resonance (NMR). Determination of structure allows for the rational design of pharmaceuticals to interact with the protein, alter protein charge configuration or charge interaction with other proteins, or to alter its function in the cell.

The invention has provided a number of genes likely to be involved in angiogenesis. As angiogenesis is critical in a number of pathological processes, the invention therefore enables therapeutic methods for the treatment of all angiogenesis-related disorders, and may enable the diagnosis or prognosis of all angiogenesis-related disorders associated with abnormalities in expression and/or function of any one of the angiogenic genes.

Examples of such disorders include, but are not limited to, cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis, cardiovascular diseases such as atherosclerosis, ischaemic limb disease and coronary artery disease.

According to another aspect of the present invention there is provided a method of treating an angiogenesis-related disorder as described above, comprising administering a selective agonist or antagonist of an angiogenic gene or protein of the invention to a subject in need of such treatment.

Still further there is provided the use of a selective agonist or antagonist of an angiogenic gene or

protein of the invention for the treatment of an angiogenesis-related disorder as described above.

For the treatment of angiogenesis-related disorders which result in uncontrolled or enhanced angiogenesis, including but not limited to, cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis and cardiovascular diseases such as atherosclerosis, therapies which inhibit the expanding vasculature are desirable. This would involve inhibition of any one of the angiogenic genes or proteins that are able to promote angiogenesis, or enhancement, stimulation or re-activation of any one of the angiogenic genes or proteins that are able to inhibit angiogenesis.

For the treatment of angiogenesis-related disorders which are characterised by inhibited or decreased angiogenesis, including but not limited to, ischaemic limb disease and coronary artery disease, therapies which enhance or promote vascular expansion are desirable. This would involve inhibition of any one of the angiogenic genes or proteins that are able to restrict angiogenesis or enhancement, stimulation or re-activation of any one of the angiogenic genes or proteins that are able to promote angiogenesis.

For instance, antisense expression of BN069 and BN096 has been shown to inhibit endothelial cell growth and proliferation. Therefore, in the treatment of disorders where angiogenesis needs to be restricted, it would be desirable to inhibit the function of these genes. Alternatively, in the treatment of disorders where angiogenesis needs to be stimulated it may be desirable to enhance the function of these genes.

For each of these cases, the relevant therapy will be useful in treating angiogenesis-related disorders regardless of whether there is a lesion in the angiogenic gene.

Inhibiting gene or protein function

Inhibiting the function of a gene or protein can be achieved in a variety of ways. Antisense nucleic acid methodologies represent one approach to inactivate genes whose altered expression is causative of a disorder. In one aspect of the invention an isolated nucleic acid molecule, which is the complement of any one of the relevant angiogenic nucleic acid molecules described above and which encodes an RNA molecule that hybridises with the mRNA encoded by the relevant angiogenic gene of the invention, may be administered to a subject in need of such treatment. Typically, a complement to any relevant one of the angiogenic genes is administered to a subject to treat or prevent an angiogenesis-related disorder.

In a further aspect of the invention there is provided the use of an isolated nucleic acid molecule which is the complement of any one of the relevant nucleic acid molecules of the invention and which encodes an RNA molecule that hybridises with the mRNA encoded by the relevant angiogenic gene of the invention, in the manufacture of a medicament for the treatment of an angiogenesis-related disorder.

Typically, a vector expressing the complement of a polynucleotide encoding any one of the relevant angiogenic genes may be administered to a subject to treat or prevent an angiogenesis-related disorder including, but not limited to, those described above. Many methods for introducing vectors into cells or tissues are available and equally suitable for use *in vivo*, *in vitro*, and *ex vivo*. For *ex vivo* therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection, by liposome injections, or by polycationic amino polymers may be achieved using methods which are well known in the art. (For example, see Goldman *et al.*, 1997).

Additional antisense or gene-targeted silencing strategies may include, but are not limited to, the use of antisense oligonucleotides, injection of antisense RNA, transfection of antisense RNA expression vectors, and the use of RNA interference (RNAi) or short interfering RNAs (siRNA). Still further, catalytic nucleic acid molecules such as DNAzymes and ribozymes may be used for gene silencing (Breaker and Joyce, 1994; Haseloff and Gerlach, 1988). These molecules function by cleaving their target mRNA molecule rather than merely binding to it as in traditional antisense approaches.

In a further aspect purified protein according to the invention may be used to produce antibodies which specifically bind any relevant angiogenic protein of the invention. These antibodies may be used directly as an antagonist or indirectly as a targeting or delivery mechanism for bringing a pharmaceutical agent to cells or tissues that express the relevant angiogenic protein. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric and single chain antibodies as would be understood by the person skilled in the art.

For the production of antibodies, various hosts including rabbits, rats, goats, mice, humans, and others may be immunized by injection with a protein of the invention or with any fragment or oligopeptide thereof, which has immunogenic properties. Various adjuvants may be used to increase immunological response and include, but are not limited to, Freund's, mineral gels such as aluminum hydroxide, and surface-active substances such as lysolecithin. Adjuvants used in humans include BCG (bacilli Calmette-Guerin) and Corynebacterium parvum.

It is preferred that the oligopeptides, peptides, or fragments used to induce antibodies to the relevant angiogenic protein have an amino acid sequence consisting of at least about 5 amino acids, and, more preferably, of at least about 10 amino acids. It is also preferable that

these oligopeptides, peptides, or fragments are identical to a portion of the amino acid sequence of the natural protein and contain the entire amino acid sequence of a small, naturally occurring molecule. Short stretches of amino acids from these proteins may be fused with those of another protein, such as KLH, and antibodies to the chimeric molecule may be produced.

Monoclonal antibodies to any relevant angiogenic protein may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique, the human B-cell hybridoma technique, and the EBV-hybridoma technique. (For example, see Kohler et al., 1975; Kozbor et al., 1985; Cote et al., 1983; Cole et al., 1984).

Monoclonal antibodies produced may include, but are not limited to, mouse-derived antibodies, humanised antibodies and fully human antibodies.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly specific binding reagents as disclosed in the literature. (For example, see Orlandi et al., 1989; Winter et al., 1991).

Antibody fragments which contain specific binding sites for any relevant angiogenic protein may also be generated. For example, such fragments include, F(ab')2 fragments produced by pepsin digestion of the antibody molecule and Fab fragments generated by reducing the disulfide bridges of the F(ab')2 fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. (For example, see Huse et al., 1989).

Various immunoassays may be used for screening to identify antibodies having the desired specificity. Numerous protocols for competitive binding or immunoradiometric assays using either polyclonal or

monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the measurement of complex formation between a protein and its specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes is preferred, but a competitive binding assay may also be employed.

In a further aspect, antagonists may include peptides, phosphopeptides or small organic or inorganic compounds. These antagonists should disrupt the function of any relevant angiogenic gene of the invention so as to provide the necessary therapeutic effect.

Peptides, phosphopeptides or small organic or inorganic compounds suitable for therapeutic applications may be identified using nucleic acids and polypeptides of the invention in drug screening applications as described below.

Enhancing gene or protein function

Enhancing, stimulating or re-activating a gene's or protein's function can be achieved in a variety of ways. In one aspect of the invention administration of an isolated nucleic acid molecule, as described above, to a subject in need of such treatment may be initiated. Typically, any relevant angiogenic gene of the invention can be administered to a subject to treat or prevent an angiogenesis-related disorder.

In a further aspect, there is provided the use of an isolated nucleic acid molecule, as described above, in the manufacture of a medicament for the treatment of an angiogenesis-related disorder.

Typically, a vector capable of expressing any relevant angiogenic gene, or a fragment or derivative thereof, may be administered to a subject to treat or prevent a disorder including, but not limited to, those described above. Transducing retroviral vectors are often used for somatic cell gene therapy because of their high

efficiency of infection and stable integration and expression. Any relevant full-length gene, or portions thereof, can be cloned into a retroviral vector and expression may be driven from its endogenous promoter or from the retroviral long terminal repeat or from a promoter specific for the target cell type of interest. Other viral vectors can be used and include, as is known in the art, adenoviruses, adeno-associated viruses, vaccinia viruses, papovaviruses, lentiviruses and retroviruses of avian, murine and human origin.

Gene therapy would be carried out according to established methods (Friedman, 1991; Culver, 1996). A vector containing a copy of any relevant angiogenic gene linked to expression control elements and capable of replicating inside the cells is prepared. Alternatively the vector may be replication deficient and may require helper cells for replication and use in gene therapy.

Gene transfer using non-viral methods of infection *in vitro* can also be used. These methods include direct injection of DNA, uptake of naked DNA in the presence of calcium phosphate, electroporation, protoplast fusion or liposome delivery. Gene transfer can also be achieved by delivery as a part of a human artificial chromosome or receptor-mediated gene transfer. This involves linking the DNA to a targeting molecule that will bind to specific cell-surface receptors to induce endocytosis and transfer of the DNA into mammalian cells. One such technique uses poly-L-lysine to link asialoglycoprotein to DNA. An adenovirus is also added to the complex to disrupt the lysosomes and thus allow the DNA to avoid degradation and move to the nucleus. Infusion of these particles intravenously has resulted in gene transfer into hepatocytes.

Although not identified to date, it is possible that certain individuals with angiogenesis-related disorders contain an abnormality in any one of the angiogenic genes of the invention. Therefore, in affected subjects that

have decreased expression or activity of an angiogenic gene, a mechanism of down-regulation may be due to abnormal methylation of promoter regions of those angiogenic genes which contain CpG islands. Therefore in an alternative approach to therapy, administration of agents that remove abnormal promoter methylation may reactivate gene expression and restore normal function to the affected cell.

In affected subjects that express a mutated form of any one of the angiogenic genes of the invention it may be possible to prevent the disorder by introducing into the affected cells a wild-type copy of the gene such that it recombines with the mutant gene. This requires a double recombination event for the correction of the gene mutation. Vectors for the introduction of genes in these ways are known in the art, and any suitable vector may be used. Alternatively, introducing another copy of the gene bearing a second mutation in that gene may be employed so as to negate the original gene mutation and block any negative effect.

In a still further aspect, there is provided a method of treating an angiogenesis-related disorder comprising administering a polypeptide, as described above, or an agonist thereof, to a subject in need of such treatment.

In another aspect the invention provides the use of a polypeptide as described above, or an agonist thereof, in the manufacture of a medicament for the treatment of an angiogenesis-related disorder. Examples of such disorders are described above.

In a further aspect, a suitable agonist may also include peptides, phosphopeptides or small organic or inorganic compounds that can mimic the function of any relevant angiogenic gene, or may include an antibody to any relevant angiogenic gene that is able to restore function to a normal level.

Peptides, phosphopeptides or small organic or inorganic compounds suitable for therapeutic applications

may be identified using nucleic acids and polypeptides of the invention in drug screening applications as described below.

In further embodiments, any of the agonists, antagonists, complementary sequences, nucleic acid molecules, proteins, antibodies, or vectors of the invention may be administered in combination with other appropriate therapeutic agents. Selection of the appropriate agents may be made by those skilled in the art, according to conventional pharmaceutical principles. The combination of therapeutic agents may act synergistically to effect the treatment or prevention of the various disorders described above. Using this approach, therapeutic efficacy with lower dosages of each agent may be possible, thus reducing the potential for adverse side effects.

Any of the therapeutic methods described above may be applied to any subject in need of such therapy, including, for example, mammals such as dogs, cats, cows, horses, rabbits, monkeys, and most preferably, humans.

Drug screening

According to still another aspect of the invention, nucleic acid molecules of the invention as well as peptides of the invention, particularly any relevant purified angiogenic polypeptides or fragments thereof, and cells expressing these are useful for screening of candidate pharmaceutical compounds in a variety of techniques for the treatment of angiogenesis-related disorders.

Still further, it provides the use wherein high throughput screening techniques are employed.

Compounds that can be screened in accordance with the invention include, but are not limited to peptides (such as soluble peptides), phosphopeptides and small organic or inorganic molecules (such as natural product or synthetic chemical libraries and peptidomimetics).

In one embodiment, a screening assay may include a cell-based assay utilising eukaryotic or prokaryotic host cells that are stably transformed with recombinant nucleic acid molecules expressing the relevant angiogenic polypeptide or fragment, in competitive binding assays. Binding assays will measure for the formation of complexes between the relevant polypeptide or fragments thereof and the compound being tested, or will measure the degree to which a compound being tested will interfere with the formation of a complex between the relevant polypeptide or fragment thereof, and its interactor or ligand.

Non cell-based assays may also be used for identifying compounds that interrupt binding between the polypeptides of the invention and their interactors. Such assays are known in the art and include for example AlphaScreen technology (PerkinElmer Life Sciences, MA, USA). This application relies on the use of beads such that each interaction partner is bound to a separate bead via an antibody. Interaction of each partner will bring the beads into proximity, such that laser excitation initiates a number of chemical reactions ultimately leading to fluorophores emitting a light signal. Candidate compounds that disrupt the binding of the relevant angiogenic polypeptide with its interactor will result in no light emission enabling identification and isolation of the responsible compound.

High-throughput drug screening techniques may also employ methods as described in WO84/03564. Small peptide test compounds synthesised on a solid substrate can be assayed through relevant angiogenic polypeptide binding and washing. The relevant bound angiogenic polypeptide is then detected by methods well known in the art. In a variation of this technique, purified angiogenic polypeptides can be coated directly onto plates to identify interacting test compounds.

An additional method for drug screening involves the use of host eukaryotic cell lines which carry mutations in

any relevant angiogenic gene of the invention. The host cell lines are also defective at the polypeptide level. Other cell lines may be used where the gene expression of the relevant angiogenic gene can be regulated (i.e. over-expressed, under-expressed, or switched off). The host cell lines or cells are grown in the presence of various drug compounds and the rate of growth of the host cells is measured to determine if the compound is capable of regulating the growth of defective cells.

The angiogenic polypeptides of the present invention may also be used for screening compounds developed as a result of combinatorial library technology. This provides a way to test a large number of different substances for their ability to modulate activity of a polypeptide. The use of peptide libraries is preferred (see WO 97/02048) with such libraries and their use known in the art.

A substance identified as a modulator of polypeptide function may be peptide or non-peptide in nature. Non-peptide "small molecules" are often preferred for many *in vivo* pharmaceutical applications. In addition, a mimic or mimetic of the substance may be designed for pharmaceutical use. The design of mimetics based on a known pharmaceutically active compound ("lead" compound) is a common approach to the development of novel pharmaceuticals. This is often desirable where the original active compound is difficult or expensive to synthesise or where it provides an unsuitable method of administration. In the design of a mimetic, particular parts of the original active compound that are important in determining the target property are identified. These parts or residues constituting the active region of the compound are known as its pharmacophore. Once found, the pharmacophore structure is modelled according to its physical properties using data from a range of sources including x-ray diffraction data and NMR. A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be added. The selection can be

made such that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, does not degrade *in vivo* and retains the biological activity of the lead compound. Further optimisation or modification can be carried out to select one or more final mimetics useful for *in vivo* or clinical testing.

It is also possible to isolate a target-specific antibody and then solve its crystal structure. In principle, this approach yields a pharmacophore upon which subsequent drug design can be based as described above. It may be possible to avoid protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analogue of the original binding site. The anti-id could then be used to isolate peptides from chemically or biologically produced peptide banks.

Another alternative method for drug screening relies on structure-based rational drug design. Determination of the three dimensional structure of the polypeptides of the invention, or the three dimensional structure of the protein complexes which may incorporate these polypeptides allows for structure-based drug design to identify biologically active lead compounds.

Three dimensional structural models can be generated by a number of applications, some of which include experimental models such as x-ray crystallography and NMR and/or from *in silico* studies using information from structural databases such as the Protein Databank (PDB). In addition, three dimensional structural models can be determined using a number of known protein structure prediction techniques based on the primary sequences of the polypeptides (e.g. SYBYL - Tripos Associated, St. Louis, MO), *de novo* protein structure design programs (e.g. MODELER - MSI Inc., San Diego, CA, or MOE - Chemical

Computing Group, Montreal, Canada) or *ab initio* methods (e.g. see US Patent Numbers 5331573 and 5579250).

Once the three dimensional structure of a polypeptide or polypeptide complex has been determined, structure-based drug discovery techniques can be employed to design biologically-active compounds based on these three dimensional structures. Such techniques are known in the art and include examples such as DOCK (University of California, San Francisco) or AUTODOCK (Scripps Research Institute, La Jolla, California). A computational docking protocol will identify the active site or sites that are deemed important for protein activity based on a predicted protein model. Molecular databases, such as the Available Chemicals Directory (ACD) are then screened for molecules that complement the protein model.

Using methods such as these, potential clinical drug candidates can be identified and computationally ranked in order to reduce the time and expense associated with typical 'wet lab' drug screening methodologies.

Compounds identified from the screening methods described above form a part of the present invention, as do pharmaceutical compositions containing these and a pharmaceutically acceptable carrier.

Pharmaceutical Preparations

Compounds identified from screening assays as indicated above can be administered to a patient at a therapeutically effective dose to treat or ameliorate a disorder associated with angiogenesis. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of the disorder.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals. The data obtained from these studies can then be used in the formulation of a range of dosages for use in humans.

Pharmaceutical compositions for use in accordance with the present invention can be formulated in a conventional manner using one or more physiological acceptable carriers, excipients or stabilisers which are well known. Acceptable carriers, excipients or stabilizers are non-toxic at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including absorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; binding agents including hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or non-ionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

The formulation of pharmaceutical compositions for use in accordance with the present invention will be based on the proposed route of administration. Routes of administration may include, but are not limited to, inhalation, insufflation (either through the mouth or nose), oral, buccal, rectal or parental administration.

Diagnostic and prognostic applications

Should abnormalities in any one of the angiogenic genes of the invention exist, which alter activity and/or expression of the gene to give rise to angiogenesis-related disorders, the polynucleotides and polypeptides of the invention may be used for the diagnosis or prognosis of these disorders, or a predisposition to such disorders. Examples of such disorders include, but are not limited to, cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis, cardiovascular diseases such as atherosclerosis, ischaemic limb disease and coronary

artery disease. Diagnosis or prognosis may be used to determine the severity, type or stage of the disease state in order to initiate an appropriate therapeutic intervention.

In another embodiment of the invention, the polynucleotides that may be used for diagnostic or prognostic purposes include oligonucleotide sequences, genomic DNA and complementary RNA and DNA molecules. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which abnormal expression or mutations in any one of the angiogenic genes may be correlated with disease. Genomic DNA used for the diagnosis or prognosis may be obtained from body cells, such as those present in the blood, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for detection of a specific sequence or may be amplified by the polymerase chain reaction (PCR) prior to analysis. Similarly, RNA or cDNA may also be used, with or without PCR amplification. To detect a specific nucleic acid sequence, direct nucleotide sequencing, reverse transcriptase PCR (RT-PCR), hybridisation using specific oligonucleotides, restriction enzyme digest and mapping, PCR mapping, RNAse protection, and various other methods may be employed. Oligonucleotides specific to particular sequences can be chemically synthesized and labelled radioactively or nonradioactively and hybridised to individual samples immobilized on membranes or other solid-supports or in solution. The presence, absence or excess expression of any one of the angiogenic genes may then be visualized using methods such as autoradiography, fluorometry, or colorimetry.

In a particular aspect, the nucleotide sequences of the invention may be useful in assays that detect the presence of associated disorders, particularly those mentioned previously. The nucleotide sequences may be labelled by standard methods and added to a fluid or

tissue sample from a patient under conditions suitable for the formation of hybridisation complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the patient sample is significantly altered in comparison to a control sample then the presence of altered levels of nucleotide sequences in the sample indicates the presence of the associated disorder. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or to monitor the treatment of an individual patient.

In order to provide a basis for the diagnosis or prognosis of an angiogenesis-related disorder associated with a mutation in any one of the angiogenic genes of the invention, the nucleotide sequence of the relevant gene can be compared between normal tissue and diseased tissue in order to establish whether the patient expresses a mutant gene.

In order to provide a basis for the diagnosis of a disorder associated with abnormal expression of any one of the angiogenic genes of the invention, a normal or standard profile for expression is established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, encoding the relevant angiogenic gene, under conditions suitable for hybridisation or amplification. Standard hybridisation may be quantified by comparing the values obtained from normal subjects with values from an experiment in which a known amount of a substantially purified polynucleotide is used. Another method to identify a normal or standard profile for expression of any one of the angiogenic genes is through quantitative RT-PCR studies. RNA isolated from body cells of a normal individual, particularly RNA isolated from endothelial cells, is reverse transcribed and real-time PCR using oligonucleotides specific for the

relevant gene is conducted to establish a normal level of expression of the gene. Standard values obtained in both these examples may be compared with values obtained from samples from patients who are symptomatic for a disorder. Deviation from standard values is used to establish the presence of a disorder.

Once the presence of a disorder is established and a treatment protocol is initiated, hybridisation assays or quantitative RT-PCR studies may be repeated on a regular basis to determine if the level of expression in the patient begins to approximate that which is observed in the normal subject. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

According to a further aspect of the invention there is provided the use of an angiogenic polypeptide as described above in the diagnosis or prognosis of an angiogenesis-related disorder associated with any one of angiogenic genes of the invention, or a predisposition to such disorders.

When a diagnostic or prognostic assay is to be based upon any relevant angiogenic polypeptide, a variety of approaches are possible. For example, diagnosis or prognosis can be achieved by monitoring differences in the electrophoretic mobility of normal and mutant proteins. Such an approach will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis or prognosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

In another aspect, antibodies that specifically bind the relevant angiogenic gene product may be used for the

diagnosis or prognosis of disorders characterized by abnormal expression of the gene, or in assays to monitor patients being treated with the relevant angiogenic gene or protein or agonists, antagonists, or inhibitors thereof. Antibodies useful for diagnostic or prognostic purposes may be prepared in the same manner as described above for therapeutics. Diagnostic or prognostic assays may include methods that utilize the antibody and a label to detect the relevant protein in human body fluids or in extracts of cells or tissues. The antibodies may be used with or without modification, and may be labelled by covalent or non-covalent attachment of a reporter molecule.

A variety of protocols for measuring the relevant angiogenic polypeptide, including ELISAs, RIAs, and FACS, are known in the art and provide a basis for diagnosing altered or abnormal levels of expression. Normal or standard values for expression are established by combining body fluids or cell extracts taken from normal mammalian subjects, preferably human, with antibody to the relevant protein under conditions suitable for complex formation. The amount of standard complex formation may be quantitated by various methods, preferably by photometric means. Quantities of protein expressed in subject, control, and disease samples from biopsied tissues are compared with the standard values. Deviation between standard and subject values establishes the parameters for diagnosing disease.

Once an individual has been diagnosed or prognosed with a disorder, effective treatments can be initiated, as described above. In the treatment of angiogenesis-related diseases which are characterised by uncontrolled or enhanced angiogenesis, the expanding vasculature needs to be inhibited. This would involve inhibiting the relevant angiogenic genes or proteins of the invention that promote angiogenesis. In addition, treatment may also need to stimulate expression or function of the relevant

angiogenic genes or proteins of the invention whose normal role is to inhibit angiogenesis but whose activity is reduced or absent in the affected individual.

In the treatment of angiogenesis-related diseases which are characterised by inhibited or decreased angiogenesis, approaches which enhance or promote vascular expansion are desirable. This may be achieved using methods essentially as described above but will involve stimulating the expression or function of the relevant angiogenic gene or protein whose normal role is to promote angiogenesis but whose activity is reduced or absent in the affected individual. Alternatively, inhibiting genes or proteins that restrict angiogenesis may also be an approach to treatment.

Microarray

In further embodiments, complete cDNAs, oligonucleotides or longer fragments derived from any of the polynucleotide sequences described herein may be used as probes in a microarray. The microarray can be used to monitor the expression level of large numbers of genes simultaneously and to identify genetic variants, mutations, and polymorphisms. This information may be used to determine gene function, to understand the genetic basis of a disorder, to diagnose or prognose a disorder, and to develop and monitor the activities of therapeutic agents. Microarrays may be prepared, used, and analysed using methods known in the art. (For example, see Schena et al., 1996; Heller et al., 1997).

Transformed hosts

The present invention also provides for the production of genetically modified (knock-out, knock-in and transgenic), non-human animal models transformed with the nucleic acid molecules of the invention. These animals are useful for the study of the function of the relevant angiogenic gene, to study the mechanisms of disease as

related to these genes, for the screening of candidate pharmaceutical compounds, for the creation of explanted mammalian cell cultures which express the protein or mutant protein and for the evaluation of potential therapeutic interventions.

Animal species which are suitable for use in the animal models of the present invention include, but are not limited to, rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs, and non-human primates such as monkeys and chimpanzees. For initial studies, genetically modified mice and rats are highly desirable due to the relative ease in generating knock-in, knock-out or transgenics of these animals, their ease of maintenance and their shorter life spans. For certain studies, transgenic yeast or invertebrates may be suitable and preferred because they allow for rapid screening and provide for much easier handling. For longer term studies, non-human primates may be desired due to their similarity with humans.

To create an animal model based on any one of the angiogenic genes of the invention, several methods can be employed. These include generation of a specific mutation in a homologous animal gene, insertion of a wild type human gene and/or a humanized animal gene by homologous recombination, insertion of a mutant (single or multiple) human gene as genomic or minigene cDNA constructs using wild type or mutant or artificial promoter elements, or insertion of artificially modified fragments of the endogenous gene by homologous recombination. The modifications include insertion of mutant stop codons, the deletion of DNA sequences, or the inclusion of recombination elements (lox p sites) recognized by enzymes such as Cre recombinase.

To create transgenic mice in order to study gain of gene function *in vivo*, any relevant angiogenic gene can be inserted into a mouse germ line using standard techniques such as oocyte microinjection. Gain of gene function can

mean the overexpression of a gene and its protein product, or the genetic complementation of a mutation of the gene under investigation. For oocyte injection, one or more copies of the wild type or mutant gene can be inserted into the pronucleus of a just-fertilized mouse oocyte. This oocyte is then reimplanted into a pseudo-pregnant foster mother. The liveborn mice can then be screened for integrants using analysis of tail DNA for the presence of the relevant human angiogenic gene sequence. The transgene can be either a complete genomic sequence injected as a YAC, BAC, PAC or other chromosome DNA fragment, a cDNA with either the natural promoter or a heterologous promoter, or a minigene containing all of the coding region and other elements found to be necessary for optimum expression.

To generate knock-out mice or knock-in mice, gene targeting through homologous recombination in mouse embryonic stem (ES) cells may be applied. Knock-out mice are generated to study loss of gene function *in vivo* while knock-in mice allow the study of gain of function or to study the effect of specific gene mutations. Knock-in mice are similar to transgenic mice however the integration site and copy number are defined in the former.

For knock-out mouse generation, gene targeting vectors can be designed such that they delete (knock-out) the protein coding sequence of the relevant angiogenic gene in the mouse genome. In contrast, knock-in mice can be produced whereby a gene targeting vector containing the relevant angiogenic gene can integrate into a defined genetic locus in the mouse genome. For both applications, homologous recombination is catalysed by specific DNA repair enzymes that recognise homologous DNA sequences and exchange them via double crossover.

Gene targeting vectors are usually introduced into ES cells using electroporation. ES cell integrants are then isolated via an antibiotic resistance gene present on the targeting vector and are subsequently genotyped to

identify those ES cell clones in which the gene under investigation has integrated into the locus of interest. The appropriate ES cells are then transmitted through the germline to produce a novel mouse strain.

In instances where gene ablation results in early embryonic lethality, conditional gene targeting may be employed. This allows genes to be deleted in a temporally and spatially controlled fashion. As above, appropriate ES cells are transmitted through the germline to produce a novel mouse strain, however the actual deletion of the gene is performed in the adult mouse in a tissue specific or time controlled manner. Conditional gene targeting is most commonly achieved by use of the cre/lox system. The enzyme cre is able to recognise the 34 base pair loxP sequence such that loxP flanked (or floxed) DNA is recognised and excised by cre. Tissue specific cre expression in transgenic mice enables the generation of tissue specific knock-out mice by mating gene targeted floxed mice with cre transgenic mice. Knock-out can be conducted in every tissue (Schwenk et al., 1995) using the 'deleter' mouse or using transgenic mice with an inducible cre gene (such as those with tetracycline inducible cre genes), or knock-out can be tissue specific for example through the use of the CD19-cre mouse (Rickert et al., 1997).

According to still another aspect of the invention there is provided the use of genetically modified non-human animals for the screening of candidate pharmaceutical compounds.

It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country. Throughout this specification and the claims, the words "comprise", "comprises" and "comprising" are used in a non-exclusive sense, except where the context requires

otherwise.

Brief Description of the Drawings

Figure 1. Examples of the classes of expression patterns of a number of angiogenic genes during angiogenesis as confirmed by Virtual Northern expression analysis. Each blot was probed with the control GAPDH1 gene to confirm loading of uniform cDNA amounts in blot construction between the defined time points of the assay.

Figure 2. Detailed Virtual Northern expression analysis of the BNO69 gene. The top panels indicate the level of expression of BNO69 at varying time points in the *in vitro* model following stimulation of human umbilical vein endothelial cells (HUVECs) with phorbol myristate acetate (PMA) plus or minus ($\alpha 2\beta 1$) antibody (AC11), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or tumour necrosis factor (TNF). The lower panel shows expression levels of BNO69 in a number of human cell lines including K562 (erythroleukaemia), KG-1a (acute myelogenous leukaemia), Jurkat (acute T cell leukaemia), HeLa (cervical adenocarcinoma), HepG2 (liver tumour), L1M12-15 (colorectal carcinoma), MDA-MB-231 (breast cancer), DU145 (prostate cancer), HEK293 (embryonic kidney), HUSMC (primary umbilical vein smooth muscle cells) ± P (PMA). HUVEC T0 and HUVEC T3 represent HUVECs harvested from the 3-D model of angiogenesis at time 0 hours and 3 hours respectively.

Figure 3. Detailed Virtual Northern expression analysis of the BNO96 gene. The top panels indicate the level of expression of BNO96 at varying time points in the *in vitro* model following stimulation of human umbilical vein endothelial cells (HUVECs) with phorbol myristate acetate (PMA) plus or minus ($\alpha 2\beta 1$) antibody (AC11), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) or tumour necrosis factor (TNF). The lower panel shows expression levels of BNO69 in

a number of human cell lines including K562 (erythroleukaemia), KG-1a (acute myelogenous leukaemia), Jurkat (acute T cell leukaemia), HeLa (cervical adenocarcinoma), HepG2 (liver tumour), L1M12-15 (colorectal carcinoma), MDA-MB-231 (breast cancer), DU145 (prostate cancer), HEK293 (embryonic kidney), HUSMC (primary umbilical vein smooth muscle cells) ± P (PMA). HUVEC T0 and HUVEC T3 represent HUVECs harvested from the 3-D model of angiogenesis at time 0 hours and 3 hours respectively.

Figure 4. BNO69 *in vitro* regulation of human umbilical vein endothelial cell (HUVEC) function using retroviral-mediated gene transfer. The proliferation of HUVECs was measured over a 3 day period by direct cell counts. The mean ± SEM is given. Over-expression of antisense BNO69 (ASBNO69R) in HUVECs inhibits their proliferation. EV: Empty vector control.

Figure 5. BNO69 *in vitro* regulation of human umbilical vein endothelial cell (HUVEC) function using adenoviral-mediated gene transfer. Over-expression of antisense BNO69 (ASBNO69A) in HUVECs leads to an inhibitory effect on cell proliferation.

Figure 6. BNO69 *in vitro* regulation of human umbilical vein endothelial cell (HUVEC) function using retroviral-mediated gene transfer. Cell morphology of endothelial cells retrovirally transfected with either empty vector (EV) control or antisense BNO69 (ASBNO69R) is shown.

Figure 7. Cell proliferation assay based on the over-expression of antisense BNO96 in human umbilical vein endothelial cells (HUVECs) using adenoviral-mediated gene transfer. Cells were infected with either vector only control (EV) or antisense BNO96 (ASBNO96), and harvested 48 hours later. Cell proliferation was measured by the colorimetric MTT assay performed 3 days after cell plating (mean ± SEM, n = 4).

Figure 8. Effect on cell migration as a result of

over-expression of antisense BNO96 in human umbilical vein endothelial cells (HUVECs) using adenoviral-mediated gene transfer. Cells were infected with either vector only control (EV) or antisense BNO96 (ASBNO96) and migration of cells towards either no agent (Nil) or the chemotactic stimulant fibronectin (Fn) was measured after 18-24 hours.

Figure 9. Effect on capillary tube formation on Matrigel as a result of over-expression of antisense BNO96 in human umbilical vein endothelial cells (HUVECs) using adenoviral-mediated gene transfer. Cells were infected with either vector only control (EV) or antisense BNO96 (ASBNO96) and assayed for tube formation over a 24 hour time period. Photos were taken after 20 hours. A and B: Low power photograph of tubes; C and D: High power photograph of tubes.

Figure 10. Effect on capillary tube formation on collagen gels as a result of over-expression of antisense BNO96 in human umbilical vein endothelial cells (HUVECs) using adenoviral-mediated gene transfer. Cells were infected with either vector only control (EV) or antisense BNO96 (ASBNO96) and assayed for tube formation over an 18-24 hour time period. Photos were taken after 3 hours as the cells were migrating through the gel. M: Migrated cell. These appear flatter and less light refractive than non-migrated cells. NM: Non-migrated cell. These cells are rounded and light refractive.

Figure 11. Effect on tumour necrosis factor (TNF)-induced E-selectin expression as a result of over-expression of antisense BNO96 in human umbilical vein endothelial cells (HUVECs) using adenoviral-mediated gene transfer. Cells were infected with either vector only control (EV) or antisense BNO96 (ASBNO96) and grown for 48 hours. TNF was added for 4 hours prior to staining for cell surface E-selectin expression using an anti E-selectin antibody. Detection was by phycoerythrin conjugated anti mouse antibody. The mean fluorescence intensity (MFI) is given.

Modes for Performing the Invention

Example 1: *In vitro* capillary tube formation

The *in vitro* model of angiogenesis is essentially as described in Gamble et al (1993). The assay was performed in collagen under the stimulation of phorbol myristate acetate (PMA) and the anti-integrin ($\alpha_2\beta_1$) antibody, RMACII. Human umbilical vein endothelial cells (HUVECs) were used in all experiments between passages 2 to 4.

Cells were harvested from bulk cultures (t=0), replated onto the collagen gels with stimulation and then harvested from the collagen gels at 0.5, 3.0, 6.0 and 24 hours after commencement of the assay. These time points were chosen since major morphological changes occur at these stages. Briefly, by 0.5 hours, cells have attached to the collagen matrix and have commenced migration into the gel. By 3.0 hours, small intracellular vesicles are visible. By 6.0 hours, these vesicles are coalescing together to form membrane bound vacuoles and the cells in the form of short sprouts have invaded the gel. After this time, these vacuoles fuse with the plasma membrane, thus expanding the intercellular space to generate the lumen (Meyer et al., 1997). The formation of these larger vacuoles is an essential requirement of lumen formation (Gamble et al., 1999). By 24 hours, the overall anastomosing network of capillary tubes has formed and has commenced degeneration.

Example 2: RNA isolation, cDNA synthesis and amplification

Cells harvested at the specified time points were used for the isolation of total RNA using the Trizol reagent (Gibco BRL) according to manufacturers conditions. SMART (Switching mechanism at 5′ end of RNA transcript) technology was used to convert small amounts of total RNA into enough cDNA to enable cDNA subtraction to be performed (see below). This was achieved using the SMART-PCR cDNA synthesis kit (Clontech-user manual PT3041-1) according to manufacturers recommendations. The SMART-PCR

cDNA synthesis protocol generated a majority of full length cDNAs which were subsequently PCR amplified for cDNA subtraction.

Example 3: Suppression subtractive hybridisation (SSH)

SSH was performed on SMART amplified cDNA in order to enrich for cDNAs that were either up-regulated or down-regulated between the cDNA populations defined by the selected time-points. This technique also allowed "normalisation" of the regulated cDNAs, thereby making low abundance cDNAs (ie poorly expressed, but important, genes) more easily detectable. To do this, the PCR-Select cDNA synthesis kit (Clontech-user manual PT3041-1) and PCR-Select cDNA subtraction kit (Clontech-user manual PT1117-1) were used based on manufacturers conditions. These procedures relied on subtractive hybridisation and suppression PCR amplification. SSH was performed between the following populations: 0 - 0.5 hours; 0.5 - 3.0 hours; 3.0 - 6.0 hours; 6.0 - 24 hours.

Example 4: Differential screening of cDNA clones

Following SSH, the cDNA fragments were digested with *EagI* and cloned into the compatible unique *NotI* site in pBluescript KS$^+$ using standard techniques (Sambrook et al., 1989). This generated forward and reverse subtracted libraries for each time period. A differential screening approach outlined in the PCR-Select Differential Screening Kit (Clontech-user manual PT3138-1) was used to identify regulated cDNAs from non-regulated ones. To do this, cDNA arrays were generated by spotting clone plasmid DNA onto nylon filters in quadruplicate. Approximately 900 individual clones were analysed by cDNA array. These arrays were subsequently probed with:

a) unsubtracted time 1 cDNA (represents mRNAs present at time 1)

b) unsubtracted time 2 cDNA (represents mRNAs present at time 2)

c) forward subtracted cDNA (represents mRNAs upregulated at time 2)

d) reverse subtracted cDNA (represents mRNAs upregulated at time 1)

All hybridisations occurred at 42°C in ExpressHyb solution (Clontech). Membranes were washed post-hybridisation according to kit instructions.

Those cDNA clones identified to be differentially expressed based on cDNA array hybridisations were subsequently sequenced. *In silico* database analysis was then used to identify homology to sequences present in the nucleotide and gene databases at the National Centre for Biotechnology Information (NCBI) in order to gain information about each clone that was sequenced. Selection of clones for further analysis was based upon the predicted function as deduced from homology searches.

Tables 1 and 2 provide information on the differentially expressed clones that were sequenced. Table 1 includes those clones which represent previously uncharacterised or novel genes, while Table 2 includes clones that correspond to previously identified genes which have not before been associated with angiogenesis. Also identified were a number of genes that have previously been shown to be involved in the process of angiogenesis. The identification of these clones provides a validation or proof of principle of the effectiveness of the angiogenic gene identification strategy employed and suggests that the clones listed in Tables 1 and 2 are additional angiogenic gene candidates.

An example from Table 1 is BN069 which encodes a novel protein of 655 amino acids. Analysis of the full-length sequence of this clone indicated the presence of a GTPase Activating Protein (GAP) domain. GAP domains are found in a class of proteins that are key regulators of

GTP binding proteins that include Ras, Rho, Cdc42 and Rac GTPases. These GTPases participate in many physiological processes which include cell motility, adhesion, cytokinesis, proliferation, differentiation and apoptosis (reviewed in Van Aelst and D'Souza-Schorey, 1997; Ridley, 2001). Rho-like GTPAses cycle between an inactive GDP bound state and an active GTP bound state. The conversion between the two forms is regulated primarily by two types of proteins. They are up-regulated by the guanine exchange factors (GEFs), which enhance the exchange of bound GDP for GTP, and down-regulated by the GTPase-activating proteins (GAPs) which increase the intrinsic rate of hydrolysis of bound GTP. When loaded with GTP, Rho GTPases gain the ability to bind a set of downstream effectors, leading for example to various cytoskeletal rearrangements.

An example from Table 2 is the BNO96 gene. Sequencing of cDNA clone 23 (BNO96) established that this clone was identical to the gamma12 subunit (GNG12) of the G protein. Heterotrimeric G proteins are involved in signal transduction from cell surface receptors to cellular effectors. The G proteins are composed of alpha (α), beta (β) and gamma (γ) subunits. Upon stimulation the α subunit dissociates from the complex and both the α and the $\beta\gamma$ subunits are able to activate multiple effectors to generate many intracellular signals.

At present 6 different β and 12 different γ subunits have been identified. Since the $\beta\gamma$ subunits are tightly associated and form highly stable dimers, they have been considered as a functional unit to date.

GNG12 has been reported to be widely expressed and rich in fibroblasts and smooth muscle cells (Ueda et al., 1999). GNG12 is a substrate for protein kinase C and is phosphorylated following stimulation with agents such as PMA, LPA (lysophosphatidic acid), growth factors and serum (Asano et al., 1998). GNG12 is also associated with F-actin (Ueda et al., 1997).

Previous reports have shown that over-expression of GNG12 alone has no effect on NIH-3T3 fibroblasts. However, over-expression of the $\beta_1\gamma_{12}$ dimer induced cell rounding, disruption of stress fibres and enhancement of cell migration. Phosphorylation of GNG12 is required for its effects on cell motility (Yasuda et al., 1998).

Based on available information regarding the BNO69 and BNO96 genes, and given that both genes have been shown to be differentially expressed during angiogenesis in the present invention, there is the suggestion that the these genes have features consistent with them performing functions associated with angiogenesis and for this reason they were analysed further.

Example 5: Virtual Northern Blot Analysis

Before functional analysis of selected clones, the differential expression observed from the cDNA array analysis of the clones listed in Tables 1 and 2 (including BNO69 and BNO96) was confirmed by Virtual Northern analysis.

Amplified cDNA from each time point was electrophoresed on an agarose/EtBr gel and the cDNA was transferred to a nylon membrane using Southern transfer according to established techniques (Sambrook et al., 1989). All cDNA clone inserts were labelled with ^{32}P using the MegaPrime DNA labelling system (Amersham Pharmacia Biotech) and hybridisations were performed in ExpressHyb solution (Clontech) according to manufacturers specifications.

Based on the results, clones were grouped according to their type of regulation pattern (Figure 1, and Tables 1 and 2). Of the 20 novel genes identified to date, 9 were confirmed to be regulated during angiogenesis, 4 gave an undetectable signal on Virtual Northern blots and the remaining clones did not indicate regulation of expression based on the Virtual Northern result. Similarly, of the 94 known genes not previously associated with angiogenesis,

59 were confirmed to be differentially regulated from the angiogenesis model. Those clones that did not display differential expression (Class F) or did not give detectable results on Virtual Northerns may still be involved in angiogenesis however further characterisation is needed.

Example 6: Cell and stimulation specificity

To further characterise the differentially expressed clones and confirm their role in angiogenesis, virtual Northern blots were again used to determine the cell type expression specificity and their stimulation in monolayer cultures with specific growth factors. Endothelial cells were plated on a 2-dimensional (2-D) collagen matrix and were stimulated for 0.5, 3.0, 6.0 and 24 hours with vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF), tumour necrosis factor α (TNFα), PMA + ACII, or PMA alone. Primary cultures of endothelial cells, fibroblasts, smooth muscle cells, together with tumour cell lines were collected. RNA was prepared from all cells and the SMART-PCR cDNA synthesis kit (Clontech-user manual PT3041-1) was used to generate cDNA for virtual Northern preparation. Prepared blots were then probed for regulation of the specific angiogenic gene of interest. Results are shown in Tables 1 and 2.

Of the clones so far analysed, all were confirmed to be expressed in endothelial cells. Of those clones listed in Table 1, three of the six clones analysed for signal specificity were shown to be influenced by the presence of VEGF, FGF and PMA. Two clones showed no response following the stimulation of endothelial cells in culture and the remaining clone showed that the differential expression was specific for the 3-dimensional and not 2-dimensional collagen gels. Of those clones listed in Table 2, two of the six clones analysed for signal specificity were shown to be influenced by the presence of VEGF and FGF and one clone was influenced by the presence of PMA only. One

clone showed no response following the stimulation of endothelial cells in culture and the remaining two clones showed that the differential expression was specific for the 3-dimensional and not 2-dimensional collagen gels.

Figures 2 and 3 provide a detailed summary of the cell and stimulation specificity results for the BNO69 and BNO96 genes respectively. These results indicate that both genes are up-regulated at the 3-hour time-point of the 3-dimensional (3-D) *in vitro* model. While the BNO69 gene is expressed in response to FGF, VEGF and PMA, expression of the BNO96 gene occurs only in response to PMA. Both genes are expressed in several cell types including endothelial cells.

Example 7: Analysis of the angiogenic genes

The genes identified by this study to be implicated in the angiogenesis process, as listed in Tables 1 and 2, may be used for further studies in order to confirm their role in angiogenesis *in vitro*. To do this, full-length coding sequences of the genes can be cloned into suitable expression vectors such as retroviruses or adenoviruses in both sense and anti-sense orientations and used for infection into endothelial cells (ECs). Retrovirus infection gives long-term EC lines expressing the gene of interest whereas adenovirus infection gives transient gene expression. Infected cells can then be subjected to a number of EC assays including proliferation and capillary tube formation to confirm the role of each gene in angiogenesis.

As an example, the effect of BNO69 and BNO96 on *in vitro* regulation of EC function has been determined and is described below.

In vitro regulation of EC function - BNO69

The effect of BNO69 on endothelial cell function and angiogenesis involved transfection of the antisense of BNO69 into endothelial cells by retroviral or adenoviral

mediated gene transfer. Human umbilical vein endothelial cells (HUVECs) at passage 1 or 2 were used for the overexpression experiments. Initially, the BNO69 gene was cloned into the replication defective retrovirus pRufNeo (Rayner and Gonda, 1994). The commercially available cell line BING was used for transfection and production of viral supernatant. HUVEC clones infected with the retrovirus and expressing the antisense BNO69 gene were selected for neo resistance using G418 and pooled together for further growth and analysis. The proliferation of the pooled clones was measured over a 3 day period by direct cell counts. Results of these experiments indicated that cells that had been infected with the antisense construct of BNO69 showed a decrease in their proliferative potential (Figure 4).

Subsequent experiments using adenoviral-mediated expression of antisense BNO69 in HUVECs showed a similar effect on cell proliferation as that observed in the retroviral system. HUVECs were infected with either vector only control or antisense BNO69 and were harvested 24 hours after infection and plated onto microtitre plates in complete growth medium. Cell proliferation was measured by the colorimetric MTT assay as described previously (Xia et al., 1999). The assay was performed 3 days after cell plating. Results of these experiments showed that the proliferation of HUVECs was inhibited by adenoviral-mediated expression of antisense BNO69 (Figure 5).

In addition, in both the retrovirus and adenovirus infection systems, a major feature of the cells infected with the antisense construct to BNO69 was the change in cell morphology. Cells appeared enlarged in size, with an increase in the extent of the cytoplasm (Figure 6). The increase in cell size was confirmed by analysis on a fluorescence activated cell sorter where a measurement of both the forward scatter and side scatter gives information on the size and granularity of the cells respectively. In both retrovirus and adenovirus systems

these parameters were changed. In retrovirus infected cells, forward scatter was 385 (EV) and 522 (ASBNO69R) while side scatter measured 289 (EV) and 508 (ASBNO69R). In the adenovirus infected cells the measurements for forward scatter were, 444 (EV) and 533 (ASBNO69R) while side scatter measured 417 (EV) and 500 (ASBNO69R).

In vitro regulation of EC function - BNO96

The effect of BNO96 on endothelial cell function and angiogenesis involved transfection of the antisense of BNO96 into endothelial cells by adenoviral gene transfer. Initially, antisense BNO96 was produced as a recombinant adenoviral plasmid employing homologous recombination in bacteria (essentially as outlined in http://coloncancer.org/protocol.htm). The resultant plasmids were transfected into the mammalian packaging cell line 293 for expansion of virus, and the virus was subsequently purified by caesium chloride gradients. Transfection efficiency was assessed by green fluorescent protein and plaque forming units as given in the protocol above.

Initially, the effect on endothelial cell proliferation of antisense BNO96 was determined. Human umbilical vein endothelial cells (HUVECs) were infected with either vector only control or antisense BNO96 and were harvested 48 hours later. Cell proliferation was measured by the colorimetric MTT assay as described previously (Xia et al., 1999). The assay was performed 3 days after cell plating (mean ± SEM, n = 4). Infection of HUVECs with antisense BNO96 was found to inhibit cell proliferation (Figure 7) when cells were cultured in full growth medium.

Another feature of the angiogenic *in vitro* model is the migration of endothelial cells into the matrix. To test the effect that BNO96 plays on this process, cell migration experiments were next conducted. Human umbilical vein endothelial cells (HUVECs) were infected with either

vector only control or antisense BNO96 and migration of cells towards either no agent or the chemotactic stimulant fibronectin was measured. The migration assay was performed as previously described (Leavesley et al., 1993). Briefly, fibronectin at 50 µg/ml was coated on the under-side of 8.0 µm Transwell filters to act as a chemotactic gradient. Cell migration was assessed after 18-24 hours. Results from these experiments showed that antisense BNO96-infected cells were inhibited from migrating towards fibronectin as a chemotactic stimulant (Figure 8).

An essential feature of the angiogenic process is the formation of capillary tubes. The role that BNO96 plays in this process was measured using the Matrigel and collagen gel models. In the Matrigel system, human umbilical vein endothelial cells (HUVECs) were infected with either vector only control or antisense BNO96 and assayed for tube formation as previously described (Cockerill et al., 1994). Briefly, 140 µl of $3X10^5$ cells/ml were plated onto the Matrigel and cell reorganisation and tube formation was assessed over a 24 hour time period. The antisense BNO96-infected cells failed to make capillary tubes in the Matrigel capillary tube assay (Figure 9).

In the collagen gel model, HUVECs were again infected with either vector only control or antisense BNO96 and assayed over an 18-24 hour time period for tube formation as previously described (Gamble et al., 1993). Expression of antisense BNO96 resulted in inhibition of cell migration (and subsequent tube formation) into the collagen gel (Figure 10).

The next experiment addressed the question of whether the inhibition of BNO96 produces endothelial cell changes that are specific for functions associated with angiogenesis. E-selectin is an endothelial specific adhesion molecule that is induced by inflammatory cytokines such as TNF and IL-1 and mediates neutrophil-endothelial cell interactions. The effect on E-selectin

expression as a result of over-expression of antisense BNO96 in human umbilical vein endothelial cells (HUVECs) was therefore determined. Methods used were as described in Litwin et al (1997). Briefly, HUVECs were infected with either vector only control or antisense BNO96 and grown for 48 hours. Following this, cells were transferred to 24-well trays and incubated overnight. Tumour necrosis factor (TNF) at 0.5 ng/ml was added for 4 hours prior to staining for cell surface E-selectin expression using an anti E-selectin antibody. Detection was by phycoerythrin conjugated anti mouse antibody. The result of these experiments showed that cells over-expressing the antisense BNO96 gene still responded in a normal fashion to the pro-inflammatory stimulant, tumour necrosis factor, to induce the adhesion molecule E-selectin (Figure 11). This suggests that the effect of antisense BNO96 on endothelial cell function is selective.

The capacity of antisense BNO96 to inhibit cell proliferation, migration and capillary tube formation but not TNF induced E-selectin expression may suggest that knockdown of the BNO96 gene specifically affects the angiogenic capacity of endothelial cells. Other cell functions such as their ability to participate in inflammatory reactions would appear to be normal (as far as those measured to date). The BNO96 gene may therefore play a defining role in the angiogenesis process and is a target for the development of therapeutics for the treatment of angiogenesis-related pathologies.

Protein interaction studies

The ability of any one of the angiogenic proteins of the invention, including BNO69 and BNO96, to bind known and unknown proteins can be examined. Procedures such as the yeast two-hybrid system are used to discover and identify any functional partners. The principle behind the yeast two-hybrid procedure is that many eukaryotic transcriptional activators, including those in yeast,

consist of two discrete modular domains. The first is a DNA-binding domain that binds to a specific promoter sequence and the second is an activation domain that directs the RNA polymerase II complex to transcribe the gene downstream of the DNA binding site. Both domains are required for transcriptional activation as neither domain can activate transcription on its own. In the yeast two-hybrid procedure, the gene of interest or parts thereof (BAIT), is cloned in such a way that it is expressed as a fusion to a peptide that has a DNA binding domain. A second gene, or number of genes, such as those from a cDNA library (TARGET), is cloned so that it is expressed as a fusion to an activation domain. Interaction of the protein of interest with its binding partner brings the DNA-binding peptide together with the activation domain and initiates transcription of the reporter genes. The first reporter gene will select for yeast cells that contain interacting proteins (this reporter is usually a nutritional gene required for growth on selective media). The second reporter is used for confirmation and while being expressed in response to interacting proteins it is usually not required for growth.

The nature of the interacting genes and proteins can also be studied such that these partners can also be targets for drug discovery.

Structural studies

Recombinant angiogenic proteins of the invention can be produced in bacterial, yeast, insect and/or mammalian cells and used in crystallographical and NMR studies. Together with molecular modeling of the protein, structure-driven drug design can be facilitated.

TABLE 1

Novel Angiogenesis Genes

Gene	Time Course Subtraction[1]	Signal Specificity[2]	Cell Type Specificity[3]	Virtual Northern[4]	Homology Details	UniGene Cluster Number	SEQ ID Numbers
BNO69	0.5-3	V/F/P	S	B	Hypothetical GAP domain containing protein	Hs 93589	1, 115
BNO71	3.0-6.0	V/F/P	M	B	LUZP – leucine zipper protein. Putative transcription factor	Hs 334673	2, 116
BNO72	0.5-3.0	3-D v.weak		B	No EST matches	None	3
BNO73	0.5-3.0	V/F/P		B	ESTs	Hs 315562	4
BNO77	0.5-3.0	NR		C	Arcbease-like protein	Hs 292812	5, 117
BNO79	0.5-0			E ?	CRELD1 (Cysteine-rich with EGF-like domains 1)	Hs 9383	6, 118
BNO82	0.5-3.0	NR	M	E ?	Hypothetical protein	Hs 172069	7, 119
BNO83	3.0-6.0			E (↑6hr)	No EST matches	None	8
BNO84	3.0-6.0			E (↓6hr)	EST	None	9
BNO85	0-0.5			undetectable	No EST matches	None	10
BNO86	0.5-3.0			undetectable	EST	None	11
BNO87	0-0.5			undetectable	No EST matches	None	12
BNO88	0-0.5			undetectable	Hypothetical protein	Hs 4863	13, 120
BNO89	0.5-3.0			F	No EST matches	None	14
BNO90	0-0.5			F	ESTs	Hs 28893	15, 121
BNO92	3.0-6.0			F	HMGE (GrpE-like protein cochaperone)	Hs 151903	16, 122
BNO94	0-0.5			F	KIAA0678	Hs 12707	17, 123
BNO95	0-0.5			F	Hypothetical protein	Hs 17283	18, 124
BNO160	0-0.5				EST, Moderately similar to GA15_HUMAN Growth arrest and DNA-damage-inducible protein GADD153 (DNA-damage inducible transcript 3) (DDIT3) (C/EBP-homologous protein) (CHOP)	Hs 335776	19
BNO174	0-0.5			L	KIAA0251	Hs 343566	20, 125

Note: [1] The time period in which isolated clones were obtained and the direction of subtraction; [2] Response to VEGF (V), bFGF (F), PMA (P) on 2-dimensional collagen gels. Response specific to 3-dimensional (3-D) not 2-dimensional collagen gels. No response (NR); [3] Expression in many (M) or several (S) cell types. [4] Specifically relates to the tube and lumen forming assay on 3-dimensional collagen. Class B: up at 3 hours; Class C: down at 0.5 hours; Class E: other regulation patterns; Class F: not regulated; Class L: Virtual Northern limited to 0 and 0.5 hour time points only, but no regulation.

TABLE 2

Genes with a Previously Unknown Role in Angiogenesis

BNO #	Time Course Subtraction[1]	Signal Specificity[2]	Cell Type Specificity[3]	Virtual Northern[4]	Homology Details	UniGene Cluster Number	SEQ ID Numbers
BNO65	0-0.5			A	SDF-2 (Stromal cell-derived factor 2)	Hs 118684	21, 126
BNO66	0-0.5			A	PRO1992 (Similar to arginyl-tRNA synthetase)	Hs 15395	22, 127
BNO67	0.5-0			A	Putative protein (Thioredoxin related protein)	Hs 6101	23, 128
BNO70	0-0.5	V/F		B	BRG1-binding protein ELD/OSA1	Hs 73287	24, 129
BNO74	3.0-6.0			B	SET domain-containing protein 7	Hs 78521	25, 130
BNO75	3.0-6.0			B ? (weak)	VPS35 (Vacuolar protein sorting 35)	Hs 264190	26, 131
BNO76	3.0-6.0			C	EBRP (Emopamil binding related protein, delta8-delta7 sterol isomerase related protein)	Hs 298490	27, 132
BNO78	0-0.5			E (↓24hr)	CPSF2 (Cleavage and polyadenylation specific factor 2)	Hs 224961	28, 133
BNO80	0.5-0			E	Hypothetical protein	Hs 323193	29, 134
BNO91	3.0-6.0			F	NADH4 (Mitochondrial gene)	None	30, 135
BNO93	0-0.5			F	SGPL1 (Sphingosine-1-phosphate lyase 1)	Hs 186613	31, 136
BNO381	0-0.5			F	COBW-like protein	Hs7535	32, 137
BNO96	0.5-0	P	S	B	GNG12 (G-coupled receptor protein γ_{12} subunit)	Hs 118520	33, 138
BNO97[5]	0-0.5			A	SDFR1 (Stromal cell-derived factor receptor 1)	Hs 6354	34, 139
BNO98	0-0.5	V/F P?		B	RYBP (Ring1 and YY1 binding protein)	Hs 7910	35, 140
BNO99[5]	0.5-0	NR		C	BMP2 (Bone morphogenic protein 2)	Hs 73853	36, 141
BNO101[5,6]	3-6, 0-0.5			A	TCEB1L (Transcription elongation factor B (SIII), polypeptide 1-like)	Hs 171626	37, 142
BNO102	0-0.5			A	PSME2 (Proteosome activator subunit 2 - PA28beta)	Hs 179774	38, 143
BNO103[5,6]	0-0.5, 3.0-6.0, 0.5-0			A	FTL (Ferritin, light polypeptide)	Hs 11134	39, 144
BNO104	3-6			A	ITCH (Itchy homolog E3 ubiquitin protein ligase)	Hs 98074	40, 145
BNO105	3-6			A	ENO1-alpha (Enolase 1 alpha)	Hs 254105	41, 146
BNO106	0.5-3			A	HNRPH2 (Heterogeneous nuclear ribonucleoprotein H2)	Hs 278857	42, 147
BNO107	0-0.5			A	UNR (NRAS-related gene)	Hs 69855	43, 148
BNO108[5,6]	0-0.5, 0.5-3.0, 6.0-24			A (↑24hr)	COX-1 (Cytochrome oxidase 1 small subunit – Mitochondrial gene)	None	44, 149
BNO111	3-6			A/(B/D)	ZFP36L2 (Zinc finger protein 36, C3H type-like 2)	Hs 78909	45, 150
BNO112	6-24			A (↑24hr)	CALM1 (Calmodulin-1)	Hs 177656	46, 151

TABLE 2 (Continued)

Genes with a Previously Unknown Role in Angiogenesis

BNO #	Time Course Subtraction[1]	Signal Specificity[2]	Cell Type Specificity[3]	Virtual Northern[4]	Homology Details	UniGene Cluster Number	SEQ ID Numbers
BNO114	6-24			A	CYP1B1 (Cytochrome P450, subfamily I (dioxin-inducible), polypeptide 1)	Hs 154654	47, 152
BNO115	3-6			A	UGTREL1 (UDP-galactose transporter related)	Hs 154073	48, 153
BNO116	0.5-3			A (↑24hr)	MCPR (Anaphase-promoting complex 1; meiotic checkpoint regulator)	Hs 40137	49, 154
BNO120	3-6			B	GLO1 (Glyoxalase 1)	Hs 75207	50, 155
BNO122	0.5-3			B ?	RPL15 (Ribosomal protein L15)	Hs 74267	51, 156
BNO123	3-6			B	SF3B1 (splicing factor 3b, subunit 1)	Hs 334826	52, 157
BNO124	0.5-3			B	AKAP12 (A kinase (PRKA) anchor protein (gravin) 12)	Hs 788	53, 158
BNO128	6-24			B	HSPA8 (Heat shock 70kD protein 8)	Hs 180414	54, 159
BNO130	0.5-3			B	LIPG (Endothelial lipase)	Hs 65370	55, 160
BNO131	0-0.5	3-D		C	PX19 like (Px19-like protein)	Hs 279529	56, 161
BNO132	0.5-0			C	PDCD6 (Programmed cell death 6)	Hs 80019	57, 162
BNO133	0.5-3			C	SDPR (Serum deprivation response - phosphatidylserine binding protein)	Hs 26530	58, 163
BNO134	0.5-0			C	GPI (Glucose phosphate isomerase)	Hs 279789	59, 164
BNO135	0.5-0			C	COX-3 (Cytochrome oxidase 3 subunit – Mitochondrial gene)	None	60, 165
BNO137	3-6	3-D	M	D	PTRF (Polymerase I and transcript release factor)	Hs 29759	61, 166
BNO140	3-6			D	CCND2 (Cyclin D2)	Hs 75586	62, 167
BNO141	0.5-0			D	GOLGA2 (Golgi autoantigen, golgin subfamily a, 2)	Hs 24049	63, 168
BNO142	3-6			E (↑6hr)	RPL11(Ribosomal protein L11)	Hs 179943	64, 169
BNO144[5]	0.5-0, 0-0.5			E (↓24hr)	EEF1A1 (Eukaryotic translation elongation factor 1 alpha 1)	Hs 181165	65, 170
BNO145	0.5-3			E (↑6hr)	ATP1A1 (ATPase, Na+/K+ transporting, alpha 1 polypeptide)	Hs 76549	66, 171
BNO146	0.5-3			E (↑6hr)	TAX1BP1 (Tax1 (human T-cell leukemia virus type I) binding protein 1)	Hs 5437	67, 172
BNO147	0.5-3			E ?	KARP-1BP3 (Ku86 Autoantigen Related Protein binding protein 3)	Hs 25132	68, 173
BNO148	3-6			E (↓24hr)	RPS6 (Ribosomal protein S6 subunit)	Hs 350166	69, 174
BNO149	6-24			E (↓6hr)	MRPL22 (Mitochondrial ribosomal protein L22)	Hs 41007	70, 175
BNO150	3-6			E (↑6hr)	BAZ2B (Bromodomain adjacent to zinc finger domain, 2B)	Hs 8383	71, 176

TABLE 2 (Continued)

Genes with a Previously Unknown Role in Angiogenesis

BNO #	Time Course Subtraction[1]	Signal Specificity[2]	Cell Type Specificity[3]	Virtual Northern[4]	Homology Details	UniGene Cluster Number	SEQ ID Numbers
BNO151	3-6			E (↑24hr)	TEGT (Testis enhanced gene transcript - BAX inhibitor 1)	Hs 74637	72, 177
BNO152	0-0.5			E (↑6hr)	TDE1 (Tumor differentially expressed 1)	Hs 272168	73, 178
BNO153	0-0.5			E (↑24hr)	RPA2 (Replication protein A2)	Hs 79411	74, 179
BNO154	0-0.5			E (↓24hr)	PABPC1 (Poly(A) binding protein, cytoplasmic 1)	Hs 172182	75, 180
BNO155	0.5-0			E	RPS13 (Ribosomal protein S13)	Hs 165590	76, 181
BNO156	0.5-0			E	TCP1 (T-complex 1)	Hs 4112	77, 182
BNO157	0.5-0			E	RNASE1 (Ribonuclease, RNase A family, 1)	Hs 78224	78, 183
BNO158	0-0.5			L	SNX5 (Sorting nexin 5)	Hs 13794	79, 184
BNO159	0.5-0			L?	NP220 (NP220 nuclear protein)	Hs 169984	80, 185
BNO161	0-0.5				DDX15 (DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 15)	Hs 5683	81, 186
BNO162	0.5-3				RPL28 (Ribosomal protein L28)	Hs 4437	82, 187
BNO163	0.5-3				UBE2L3 (Ubiquitin-conjugating enzyme E2L 3)	Hs 108104	83, 188
BNO164	3-6				CYTB (Cytochrome b – Mitochondrial gene)	None	84, 189
BNO166	0-0.5			?	MPHOSPH6 (M-phase phosphoprotein 6)	Hs 152720	85, 190
BNO167[5]	0-0.5				SSBP2 (Single-stranded DNA binding protein 2)	Hs 169833	86, 191
BNO168	0.5-0				NADH1 (NADH dehydrogenase subunit 1 – Mitochondrial gene)	None	87, 192
BNO169	0.5-3			U	PHF3 (PHD finger protein 3)	Hs 78893	88, 193
BNO170[5]	0-0.5, 0.5-0			U	ICMT (Isoprenylcysteine carboxyl methyltransferase)	Hs 183212	89, 194
BNO171	0-0.5			L	HCFC1 (Host cell factor C1 - VP16-accessory protein)	Hs 83634	90, 195
BNO173	0-0.5			L	QKI7/7B (QKI Homolog of mouse quaking QKI - KH domain RNA binding protein)	Hs 15020	91, 196
BNO175	0.5-0			L	S100A13 (S100 calcium binding protein A13)	Hs 14331	92, 197
BNO176	0.5-0			L?	CGI-99 protein	Hs 110803	93, 198
BNO177	0-0.5			L?	EXT1 (Exostoses (multiple) 1)	Hs 184161	94, 199
BNO179	0.5-0				TI-227H (Mitochondrial gene)	None	95
BNO180	0.5-0				KPNA2 (Karyopherin alpha 2 (RAG cohort 1 - importin alpha 1)	Hs 159557	96, 200
BNO181[5]	0.5-3				RPS9 (Ribosomal protein S9)	Hs 180920	97, 201
BNO182	3-6				NCBP2 (Nuclear cap binding protein subunit 2)	Hs 240770	98, 202
BNO183	3-6				S12 rRNA (Mitochondrial gene)	None	99

TABLE 2 (Continued)

Genes with a Previously Unknown Role in Angiogenesis

BNO #	Time Course Subtraction[1]	Signal Specificity[2]	Cell Type Specificity[3]	Virtual Northern[4]	Homology Details	UniGene Cluster Number	SEQ ID Numbers
BNO366	3-6			F	ATP synthase 6 (Mitochondrial gene)	None	100, 203
BNO367	3-6			F	HSP105 (Heat shock protein 105)	Hs 36927	101, 204
BNO368	0.5-0			L?	PROX1 (Prospero-related homeobox 1)	Hs 159437	102, 205
BNO369[5]	6-24, 0.5-0			C/F?	ACTB (actin, beta)	Hs 288061	103, 206
BNO370	6-24			F	TMSB4X (Thymosin, beta 4 X chromosome)	Hs 75968	104, 207
BNO371[5]	3-6, 0.5-0			F	16S rRNA (Mitochondrial gene)	None	105
BNO373	3-6			F	APLP2 (Amyloid beta (A4) precursor-like protein 2)	Hs 279518	106, 208
BNO374	0-0.5			F	EPLIN beta (Epithelial protein lost in neoplasm beta)	Hs 10706	107, 209
BNO375	0-0.5			F	EIF3S9 (Eukaryotic translation initiation factor 3 subunit 9)	Hs 57783	108, 210
BNO376	0.5-0			F	PSMC1 (Proteasome 26S subunit, ATPase, 1)	Hs 4745	109, 211
BNO377	0-0.5			F	TCTA (T-cell leukemia translocation altered gene)	Hs 250894	110, 212
BNO378	0.5-0			F	NTF2 (Nuclear transport factor 2)	Hs 151734	111, 213
BNO379	3-6			F	MLC-B (Myosin regulatory light chain)	Hs 180224	112, 214
BNO380	0-0.5			F	CHRNA1 (nicotinic acetylcholine receptor alpha 1 subunit)	Hs 2266	113, 215
BNO382	0-0.5			F	MAP1B (Microtubule associated protein 1B)	Hs 103042	114, 216

Note: [1] The time period in which isolated clones were obtained and the direction of subtraction; [2] Response to VEGF (V), bFGF (F), PMA (P) on 2-dimensional collagen gels. [4] Response specific to 3-dimensional (3-D) not 2-dimensional collagen gels. No response (NR). [3] Expression in several (S) cell types; expression in many (M) cell types. Specifically relates to the tube and lumen forming assay on 3-dimensional collagen. Class A: up at 0.5 hours; Class B: up at 3 hours; Class C: down at 0.5 hours; Class D: down at 3 hours; Class E: Other regulation patterns; Class F: not regulated; Class L: Virtual Northern limited to 0 and 0.5 hour time points only, but no regulation. [5] Multiple cDNA clones were identified for this BNO gene. [6] The multiple cDNA clones identified for this BNO gene showed regulation of expression at more than one time point of the angiogenesis model.

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Claims

1. An isolated nucleic acid molecule comprising the sequence set forth in one of SEQ ID Numbers: 1 to 20.

2. An isolated nucleic acid molecule comprising the sequence set forth in one of SEQ ID Numbers: 1 to 114, or a fragment thereof, and which encodes a polypeptide that plays a role in an angiogenic process.

3. An isolated nucleic acid molecule that is at least 70% identical to a nucleic acid molecule comprising the sequence set forth in one of SEQ ID Numbers: 1 to 114, and which encodes a polypeptide that plays a role in an angiogenic process.

4. An isolated nucleic acid molecule as claimed in claim 3 that is at least 85% identical.

5. An isolated nucleic acid molecule as claimed in claim 3 that is at least 95% identical.

6. An isolated nucleic acid molecule as claimed in any one of claims 3 to 5 wherein sequence identity is determined using the BLAST algorithm with the BLOSUM 62 default matrix.

7. An isolated nucleic acid molecule that encodes a polypeptide that plays a role in an angiogenic process, and which hybridizes under stringent conditions with a nucleic acid molecule comprising the nucleotide sequence set forth in one of SEQ ID Numbers: 1 to 114.

8. An isolated nucleic acid molecule as claimed in claim 7 wherein the stringent conditions comprise hybridization at 42°C in 750 mM NaCl, 75 mM trisodium citrate, 2% SDS, 50% formamide, 1X Denhart's, 10% w/v) dextran sulphate and 100 ug/ml denatured salmon sperm DNA.

9. An isolated nucleic acid molecule as claimed in any one of claims 1 to 8, which encodes a polypeptide that plays a role in diseases associated with angiogenesis including but not restricted to cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis, cardiovascular diseases such as atherosclerosis, ischaemic limb disease and coronary artery disease.

10. An isolated nucleic acid molecule consisting any one of the nucleotide sequences set forth in SEQ ID Numbers: 1 to 20.

11. Use of a nucleic acid molecule selected from the group consisting of DNA molecules having the sequence set forth in SEQ ID Numbers: 70, 72 to 73, 78, 83 to 87, 89, 160 or 174 to identify and/or obtain full-length human genes involved in an angiogenic process.

12. Use as claimed in claim 11 wherein a full-length human gene is identified by *in silico* database analysis.

13. Use as claimed in either one of claims 11 or 12 wherein additional sequence is obtained using hybridisation with one or more of said nucleotide molecules, inverse PCR, restriction site PCR, PCR walking techniques or RACE.

14. A gene when identified by the use of a DNA molecule selected from any one of SEQ ID Numbers: 70, 72 to 73, 78, 83 to 87, 89, 160 or 174.

15. An isolated polypeptide comprising the sequence set forth in one of SEQ ID Numbers: 115 to 125.

16. An isolated polypeptide comprising the sequence set forth in one of SEQ ID Numbers: 115 to 217, or a fragment thereof, that plays a role in an angiogenic process.

17. An isolated polypeptide that plays a role in an angiogenic process, and having at least 70% identity with the amino acid sequence set forth in SEQ ID Numbers: 115 to 217.

18. An isolated polypeptide as claimed in claim 17 with at least 85% sequence identity.

19. An isolated polypeptide as claimed in claim 17 with at least 95% sequence identity.

20. An isolated polypeptide as claimed in any one of claims 17 to 19 wherein sequence identity is determined using the BLAST algorithm with the BLOSUM 62 default matrix.

21. An isolated polypeptide as claimed in any one of claims 15 to 20 that plays a role in diseases associated with an angiogenic process including but not restricted to cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis, cardiovascular diseases such as atherosclerosis, ischaemic limb disease and coronary artery disease.

22. An isolated polypeptide consisting any one of the amino acid sequences set forth in SEQ ID Numbers: 115 to 125.

23. An isolated polypeptide complex that plays a role in an angiogenic process, said polypeptide complex comprising a polypeptide as defined in any one of claims 15 to 22.

24. An expression vector comprising a nucleic acid molecule as defined in any one of claims 1 to 10.

25. A cell transformed with an expression vector of claim 24.

26. A cell as claimed in claim 25 which is an eukaryotic cell.

27. A method of preparing a polypeptide comprising the steps of :

(1) culturing cells as claimed in either one of claims 25 to 26 under conditions effective for polypeptide production; and

(2) harvesting the polypeptide.

28. A polypeptide prepared by the method of claim 27.

29. A method of modulating angiogenesis comprising modulating the expression or activity of a polypeptide in a cell, wherein the polypeptide is encoded by a nucleic acid sequence as claimed in any one of claims 1 to 10.

30. The method of claim 29 wherein the nucleic acid sequence is selected from the group consisting of SEQ ID Numbers: 1 to 20.

31. The method of claim 29 wherein the polypeptide comprises an amino acid sequence as claimed in any one of claims 15 to 22, or an active fragment thereof.

32. The method of claim 31 wherein the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID Numbers: 115 to 125.

33. The method of claim 29 wherein the polypeptide forms part of a polypeptide complex.

34. The method of claim 29 wherein the expression or activity of the polypeptide is modulated by introducing

into the cell an antagonist or agonist of a nucleic acid molecule as defined in any one of claims 1 to 10 or a polypeptide as claimed in any one of claims 15 to 22 or claim 27.

35. The method of claim 29 wherein the expression or activity of the polypeptide is modulated by introducing into the cell an antisense to an isolated nucleic acid molecule as claimed in any one of claims 1 to 10.

36. The method of claim 29 wherein the expression or activity of the polypeptide is modulated by introducing into the cell a nucleic acid molecule which is the complement of at least a portion of a nucleic acid sequence as claimed in any one of claims 1 to 10 and is capable of modulating expression or levels of said nucleic acid sequence.

37. The method of claim 36 wherein the nucleic acid molecule is an RNA molecule that hybridizes with the mRNA encoded by a nucleic acid sequence as claimed in any one of claims 1 to 10.

38. The method of claim 36 wherein the nucleic acid molecule is a catalytic nucleic acid molecule that is targeted to a nucleic acid sequence as claimed in any one of claims 1 to 10.

39. The method of claim 38 wherein the catalytic nucleic acid molecule is a DNAzyme.

40. The method of claim 38 wherein the catalytic nucleic acid molecule is a ribozyme.

41. The method of claim 29 wherein the polypeptide expression or activity is modulated by an antibody capable of binding the polypeptide.

42. The method of claim 41 wherein the antibody is a fully human antibody.

43. The method of claim 41 wherein the antibody is selected from the group consisting of
a monoclonal antibody, a humanised antibody, a chimaeric antibody or an antibody fragment including a Fab fragment, $(Fab')_2$ fragment, Fv fragment, single chain antibodies and single domain antibodies.

44. The method of claim 29 wherein the polypeptide expression or activity is modulated by introducing into the cell a nucleic acid molecule comprising a nucleic acid sequence as claimed in any one of claims 1 to 10, or an active fragment or variant thereof.

45. The method of claim 44 wherein the nucleic acid molecule is introduced by way of an expression vector as claimed in claim 24.

46. The method of claim 29 wherein the polypeptide expression or activity is modulated by introducing into the cell a polypeptide comprising an amino acid sequence as claimed in any one of claims 15 to 22 or claim 27.

47. The method of any one of claims 29 to 46 wherein angiogenesis is uncontrolled or enhanced.

48. The method of any one of claims 29 to 46 wherein angiogenesis is inappropriately arrested or decreased.

49. A method for the treatment of an angiogenesis-related disorder, comprising modulating the expression or activity of a polypeptide encoded by a nucleic acid sequence as claimed in any one of claims 1 to 10.

50. The method of claim 49 wherein the nucleic acid sequence is selected from the group consisting of SEQ ID Numbers: 1 to 20.

51. The method of claim 49 wherein the polypeptide comprises an amino acid sequence as claimed in any one of claims 15 to 22, or an active fragment thereof.

52. The method of claim 51 wherein the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID Numbers: 115 to 125.

53. The method of claim 49 wherein the polypeptide forms part of a polypeptide complex.

54. The method of claim 49 wherein the expression or activity of the polypeptide is modulated by introducing into the cell an antagonist or agonist of a nucleic acid molecule as defined in any one of claims 1 to 10 or an antagonist or agonist of a polypeptide as claimed in any one of claims 15 to 22 or claim 27.

55. The method of claim 49 wherein the expression or activity of the polypeptide is modulated by introducing into the cell an antisense to an isolated nucleic acid molecule as claimed in any one of claims 1 to 10.

56. The method of claim 49 wherein the expression or activity of the polypeptide is modulated by introducing into the cell a nucleic acid molecule which is the complement of at least a portion of a nucleic acid sequence as claimed in any one of claims 1 to 10 and is capable of modulating expression or levels of said nucleic acid sequence.

57. The method of claim 56 wherein the nucleic acid molecule is an RNA molecule that hybridizes with the mRNA

encoded by a nucleic acid sequence as claimed in any one of claims 1 to 10.

58. The method of claim 56 wherein the nucleic acid molecule is a catalytic nucleic acid molecule that is targeted to a nucleic acid sequence as claimed in any one of claims 1 to 10.

59. The method of claim 58 wherein the catalytic nucleic acid molecule is a DNAzyme.

60. The method of claim 58 wherein the catalytic nucleic acid molecule is a ribozyme.

61. The method of claim 49 wherein the polypeptide expression or activity is modulated by an antibody capable of binding the polypeptide.

62. The method of claim 61 wherein the antibody is a full human antibody.

63. The method of claim 61 wherein the antibody is selected from the group consisting of a monoclonal antibody, a humanised antibody, a chimaeric antibody or an antibody fragment including a Fab fragment, $(Fab')_2$ fragment, Fv fragment, single chain antibodies and single domain antibodies.

64. The method of claim 49 wherein the polypeptide expression or activity is modulated by introducing into the cell a nucleic acid molecule comprising a nucleic acid sequence as claimed in any one of claims 1 to 10, or an active fragment or variant thereof.

65. The method of claim 64 wherein the nucleic acid molecule is introduced by way of an expression vector as claimed in claim 24.

66. The method of claim 49 wherein the polypeptide expression or activity is modulated by introducing into the cell a polypeptide comprising an amino acid sequence as claimed in any one of claims 15 to 22 or claim 27.

67. The method of any one of claims 49 to 66 wherein the angiogenesis-related disorder involves uncontrolled or enhanced angiogenesisis, or is a disorder in which a decreased vasculature is of benefit.

68. The method of claim 67 wherein the disorder is selected from the group consisting of cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis and cardiovascular diseases such as atherosclerosis.

69. The method of any one of claims 49 to 66 wherein the angiogenesis-related disorder involves inappropriately arrested or decreased angiogenesisis, or is a disorder in which an expanding vasculature is of benefit.

70. The method of claim 69 wherein the disorder is selected from the group consisting of ischaemic limb disease or coronary artery disease.

71. Use of a modulator of expression or activity of a polypeptide encoded by a nucleic acid sequence as claimed in any one of claims 1 to 10 in the manufacture of a medicament for the treatment of an angiogenesis-related disorder.

72. The use of claim 71 wherein the nucleic acid sequence is selected from the group consisting of SEQ ID Numbers: 1 to 20.

73. The use of claim 71 wherein the polypeptide comprises an amino acid sequence as claimed in any one of claims 15 to 22, or an active fragment thereof.

74. The use of claim 73 wherein the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID Numbers: 115 to 125.

75. The use of claim 71 wherein the polypeptide forms part of a polypeptide complex.

76. The use of claim 71 wherein the expression or activity of the polypeptide is modulated by introducing into the cell an antagonist or agonist of a nucleic acid molecule as defined in any one of claims 1 to 10 or an antagonist or agonist of a polypeptide as claimed in any one of claims 15 to 22 or claim 27.

77. The use of claim 71 wherein the expression or activity of the polypeptide is modulated by introducing into the cell an antisense to an isolated nucleic acid molecule as claimed in any one of claims 1 to 10.

78. The use of claim 71 wherein the expression or activity of the polypeptide is modulated by introducing into the cell a nucleic acid molecule which is the complement of at least a portion of a nucleic acid sequence as claimed in any one of claims 1 to 10 and is capable of modulating expression or levels of said nucleic acid sequence.

79. The use of claim 78 wherein the nucleic acid molecule is an RNA molecule that hybridizes with the mRNA encoded by a nucleic acid sequence as claimed in any one of claims 1 to 10.

80. The use of claim 78 wherein the nucleic acid molecule is a catalytic nucleic acid molecule that is targeted to a nucleic acid sequence as claimed in any one of claims 1 to 10.

81. The use of claim 80 wherein the catalytic nucleic acid molecule is a DNAzyme.

82. The use of claim 80 wherein the catalytic nucleic acid molecule is a ribozyme.

83. The use of claim 71 wherein the polypeptide expression or activity is modulated by an antibody capable of binding the polypeptide.

84. The use of claim 83 wherein the antibody is a full human antibody.

85. The use of claim 83 wherein the antibody is selected from the group consisting of a monoclonal antibody, a humanised antibody, a chimaeric antibody or an antibody fragment including a Fab fragment, $(Fab')_2$ fragment, Fv fragment, single chain antibodies and single domain antibodies.

86. The use of claim 71 wherein the polypeptide expression or activity is modulated by introducing into the cell a nucleic acid molecule comprising a nucleic acid sequence as claimed in any one of claims 1 to 10, or an active fragment or variant thereof.

87. The use of claim 86 wherein the nucleic acid molecule is introduced by way of an expression vector as claimed in claim 24.

88. The use of claim 71 wherein the polypeptide expression or activity is modulated by introducing into the cell a polypeptide comprising an amino acid sequence as claimed in any one of claims 15 to 22 or claim 27.

89. The use of any one of claims 71 to 88 wherein the angiogenesis-related disorder involves uncontrolled or

enhanced angiogenesisis, or is a disorder in which a decreased vasculature is of benefit.

90. The use of claim 89 wherein the disorder is selected from the group consisting of cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis and cardiovascular diseases such as atherosclerosis.

91. The use of any one of claims 71 to 88 wherein the angiogenesis-related disorder involves inappropriately arrested or decreased angiogenesisis, or is a disorder in which an expanding vasculature is of benefit.

92. The use of claim 91 wherein the disorder is selected from the group consisting of ischaemic limb disease or coronary artery disease.

93. The use of a nucleic acid molecule as claimed in any one of claims 1 to 10 for the screening of candidate pharmaceutical compounds useful in the treatment of angiogenesis-related disorders.

94. A compound useful in the treatment of angiogenesis-related disorders when identified by the use of a nucleic acid molecule as claimed in any one of claims 1 to 10.

95. The use of a polypeptide as claimed in any one of claims 15 to 22 or claim 27, or a polypeptide complex as claimed in claim 23 for the screening of candidate pharmaceutical compounds useful in the treatment of angiogenesis-related disorders.

96. A compound useful in the treatment of angiogenesis-related disorders when identified by the use of a polypeptide as claimed in any one of claims 15 to 22 or claim 27, or a polypeptide complex as claimed in claim 23.

97. The use of a cell as claimed in either one of claims 25 or 26 for the screening of candidate pharmaceutical compounds useful in the treatment of angiogenesis-related disorders.

98. A compound useful in the treatment of angiogenesis-related disorders when identified by the use of a cell as claimed in either one of claims 25 or 26.

99. A method of screening for a candidate pharmaceutical compound useful in the treatment of angiogenesis-related disorders comprising the steps of:

(1) providing a polypeptide as claimed in any one of claims 15 to 22 or claim 27, or a polypeptide complex as claimed in claim 23;

(2) adding a candidate pharmaceutical compound to said polypeptide; and

(3) determining the binding of said candidate compound to said polypeptide;

wherein a compound that binds to the polypeptide or polypeptide complex is a candidate pharmaceutical compound.

100. A method of screening for candidate pharmaceutical compound useful in the treatment of angiogenesis-related disorders comprising the steps of:

(1) providing a cell, as claimed in either one of claims 25 or 26;

(2) adding a candidate pharmaceutical compound to said cell; and

(3) determining the effect of said candidate pharmaceutical compound on the functional properties of said cell;

wherein a compound that alters the functional properties of said cell is a candidate pharmaceutical compound.

101. A method of screening for a candidate pharmaceutical compound useful in the treatment of angiogenesis-related disorders comprising the steps of:

(1) providing a cell, as claimed in either one of claims 25 or 26;

(2) adding a candidate pharmaceutical compound to said cell; and

(3) determining the effect of said candidate pharmaceutical compound on the expression of the nucleic acid molecule that is part of the expression vector in said cell;

wherein a compound that alters the expression of the nucleic acid molecule that is part of the expression vector in said cell is a candidate pharmaceutical compound.

102. A method of screening for a candidate pharmaceutical compound useful in the treatment of angiogenesis-related disorders comprising the steps of:

(1) providing a cell, as claimed in either one of claims 25 or 26;

(2) adding a candidate pharmaceutical compound to said cell; and

(3) determining the effect of said candidate pharmaceutical compound on the expression or activity of the polypeptide encoded by the nucleic acid molecule that is part of the expression vector in said cell;

wherein a compound that alters the expression or activity of polypeptide encoded by the nucleic acid molecule that is part of the expression vector in said cell is a candidate pharmaceutical compound.

103. A compound when identified by the method of any one of claims 100 to 102.

104. A pharmaceutical composition comprising a compound as claimed in 103 and a pharmaceutically acceptable carrier.

105. An antibody which is immunologically reactive with an isolated polypeptide as claimed in claim 15.

106. An antibody as claimed in claim 105 which is a fully human antibody.

107. An antibody as claimed in claim 105 which is selected from the group consisting of a monoclonal antibody, a humanised antibody, a chimaeric antibody or an antibody fragment including a Fab fragment, $(Fab')_2$ fragment, Fv fragment, single chain antibodies and single domain antibodies.

108. A catalytic nucleic acid targeted to a nucleic acid sequence as claimed in claim 1.

109. A catalytic nucleic acid of claim 108 which is a DNAzyme.

110. A catalytic nucleic acid of claim 108 which is a ribozyme.

111. Use of a nucleic acid molecule as claimed in any one of claims 1 to 10 in the diagnosis or prognosis of an angiogenesis-related disorder.

112. Use of a polypeptide as claimed in any one of claims 15 to 22 or claim 27 in the diagnosis or prognosis of an angiogenesis-related disorder.

113. Use of an antibody as claimed in any one of claims 105 to 107 or an antibody to a polypeptide as claimed in claim 16 in the diagnosis or prognosis of an angiogenesis-related disorder.

114. A method for the diagnosis of an angiogenesis-related disorder comprising the steps of:

(1) establishing a profile for normal expression and/or activity of a gene as claimed in any one of claims 1 to 10, in unaffected subjects;

(2) measuring the level of expression and/or activity of the gene in a person suspected of abnormal expression and/or activity of the gene; and

(3) comparing the measured level of expression and/or activity with the profile for normal expression and/or activity;

wherein an altered level of expression and/or activity in said subject is an indication of an angiogenesis-related disorder, or a predisposition thereto.

115. A method as claimed in claim 114 wherein reverse transcriptase PCR is employed to measure levels of expression.

116. A method as claimed in claim 114 wherein a hybridisation assay using a probe derived from the gene, or a fragment thereof, is employed to measure levels of expression.

117. A method for the diagnosis of an angiogenesis-related comprising the steps of:

(1) obtaining DNA from a subject corresponding to a nucleic acid sequence as claimed in any one of claims 1 to 10; and

(2) comparing the DNA from said to the DNA of the corresponding wild-type gene;

wherein altered DNA properties in said subject is an indication of an angiogenesis-related disorder, or a predisposition thereto.

118. A method as claimed in claim 117 wherein the DNA of the gene is sequenced and the sequences compared.

119. A method as claimed in claim 117 wherein the DNA of the gene is subjected to SSCP analysis.

120. A method for the diagnosis of an angiogenesis-related disorder comprising the steps of:

(1) establishing a physical property of a wild-type polypeptide as claimed in any one of claims 15 to 22;

(2) obtaining the polypeptide from a person suspected of an abnormality of that polypeptide; and;

(3) measuring the property for the polypeptide expressed by the person and comparing it to the established property for wild-type polypeptide;

wherein altered polypeptide properties in said subject is an indication of an angiogenesis-related disorder, or a predisposition thereto.

121. A method as claimed in claim 120 wherein the property is the electrophoretic mobility.

122. A method as claimed in claim 120 wherein the property is the proteolytic cleavage pattern.

123. A genetically modified non-human animal transformed with an isolated nucleic acid molecule as defined in any one of claims 1 to 10.

124. A genetically modified non-human animal as claimed in claim 123 in which the animal is selected from the group consisting of rats, mice, hamsters, guinea pigs, rabbits, dogs, cats, goats, sheep, pigs and non-human primates such as monkeys and chimpanzees.

125. A genetically modified non-human animal as claimed in claim 123 wherein the animal is a mouse.

126. Use of a genetically modified non-human animal as defined in any one of claims 123 to 125 in screening for candidate pharmaceutical compounds useful for the treatment of angiogenesis-related disorders.

127. The use of any one of claims 93 to 98 or claim 126 wherein the angiogenesis-related disorder involves uncontrolled or enhanced angiogenesisis, or is a disorder in which a decreased vasculature is of benefit.

128. The use of claim 127 wherein the disorder is selected from the group consisting of cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis and cardiovascular diseases such as atherosclerosis.

129. The use of any one of claims 93 to 98 or claim 126 wherein the angiogenesis-related disorder involves inappropriately arrested or decreased angiogenesisis, or is a disorder in which an expanding vasculature is of benefit.

130. The use of claim 129 wherein the disorder is selected from the group consisting of ischaemic limb disease or coronary artery disease.

PCT/AU02/01282

Figure 1

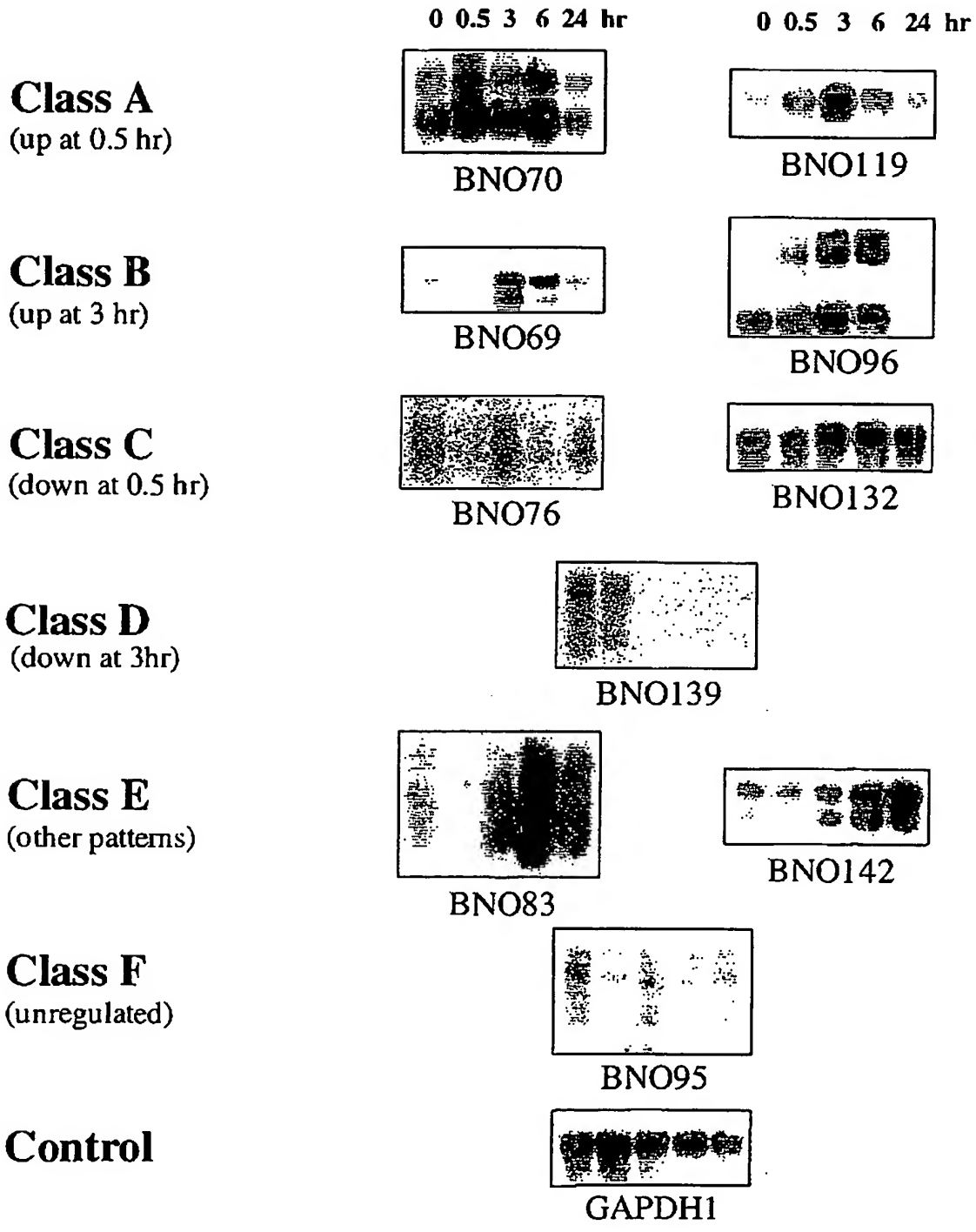
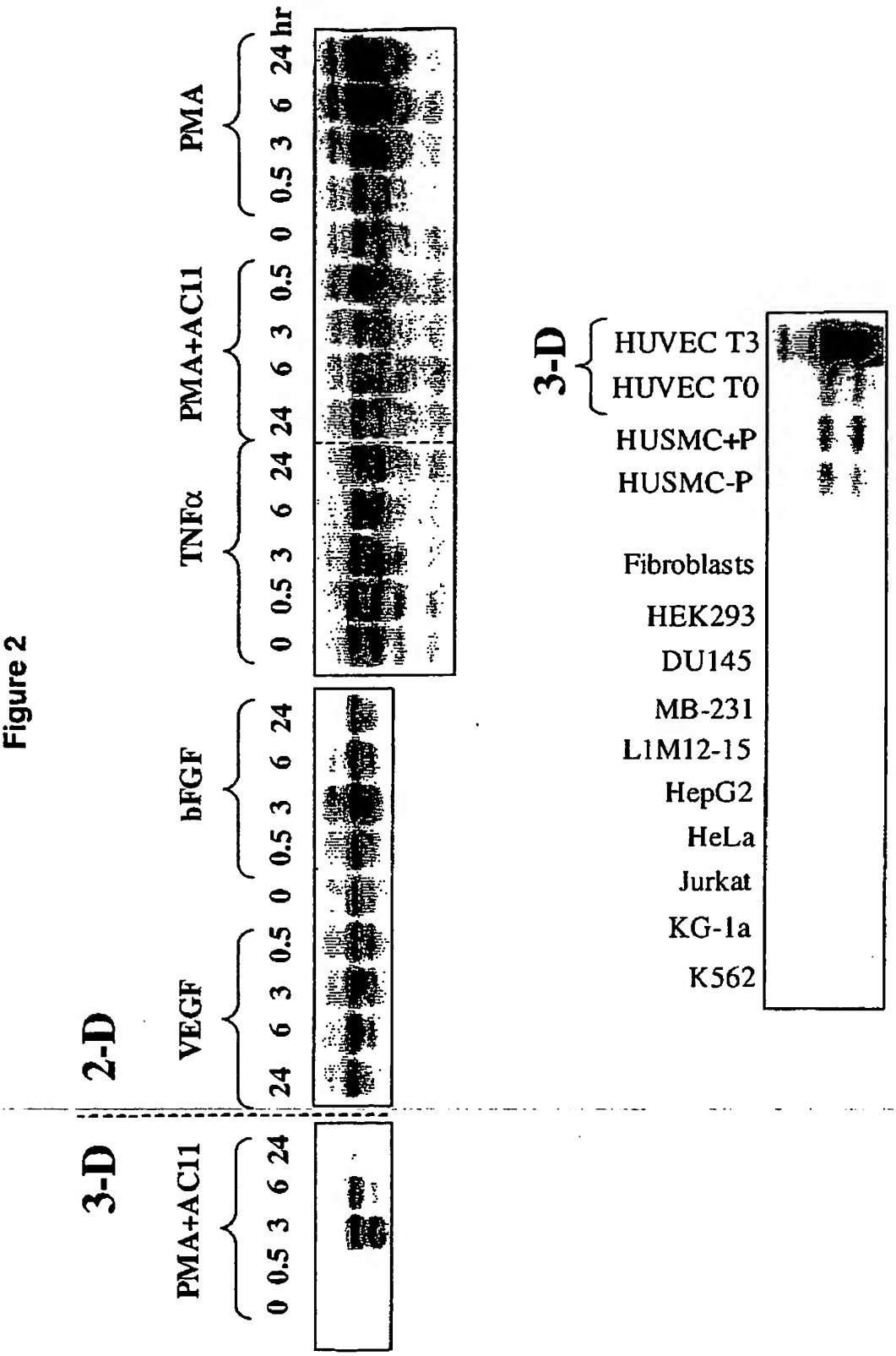


Figure 2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bFGF
0 0.5 3 6 24
TNFα
0 0.5 3 6 24
PMA+AC11
0 0.5 3 6 24
PMA
0 0.5 3 6 24 hr
3-D
PMA+AC11
0 0.5 3 6 24
3-D
HUVEC T3
HUVEC T0
HUSMC+P
HUSMC-P
Fibroblasts
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L1M12-15
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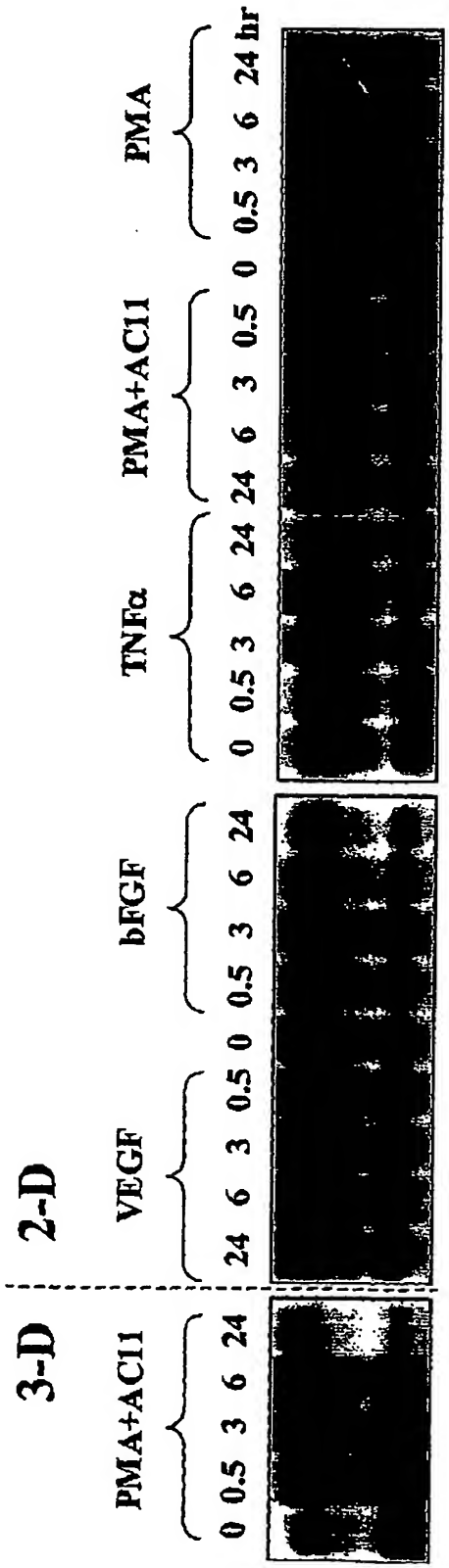
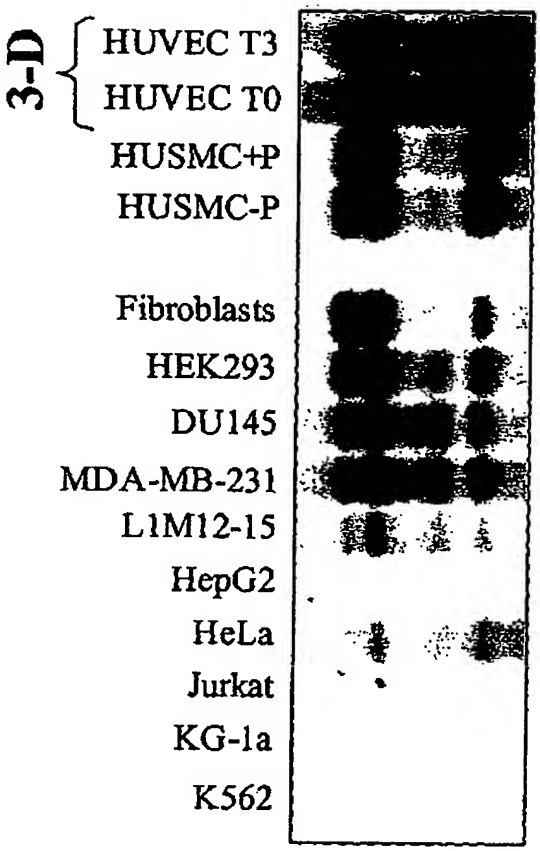
HeLa
Jurkat
KG-1a
K562

PCT/AU02/01282

Figure 3





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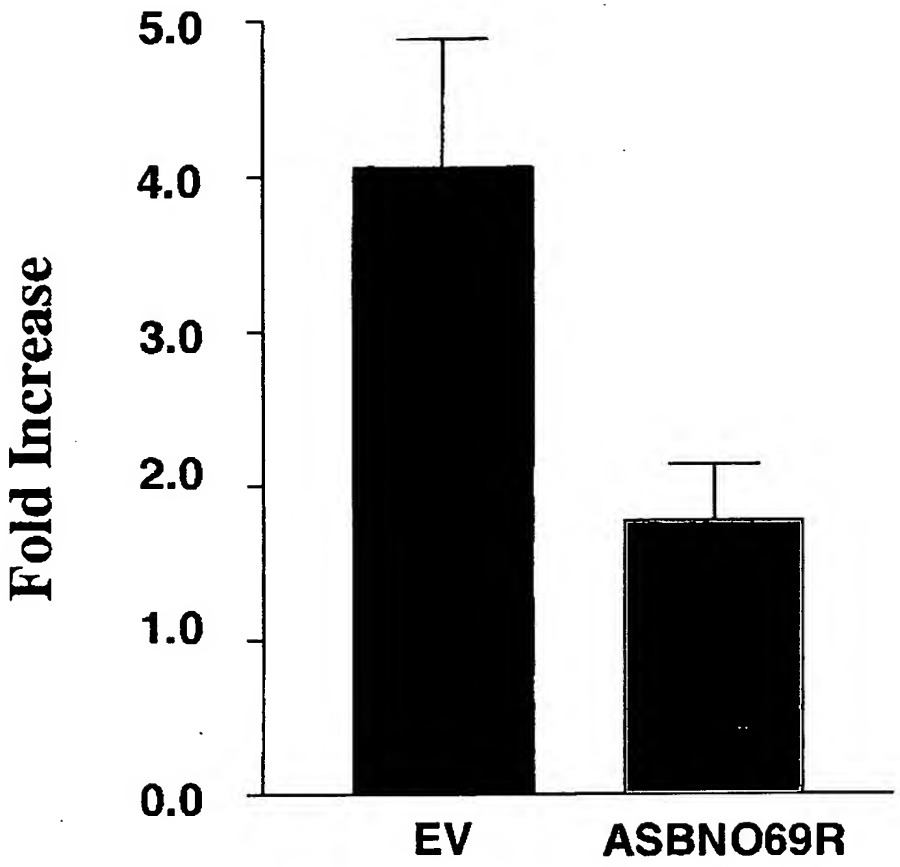
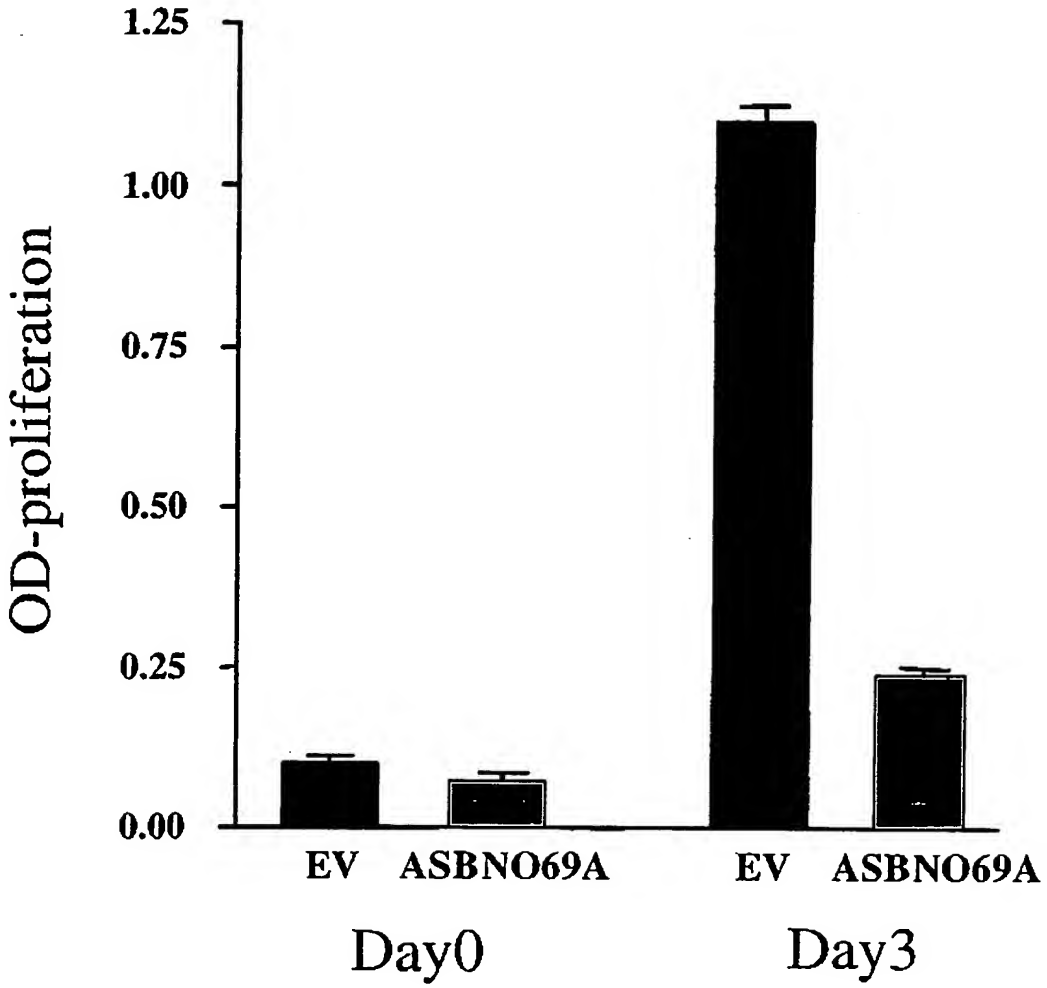


Figure 5


1.25
1.00
0.75
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0.25
0.00
OD-proliferation
EV
ASBNO69A
EV
ASBNO69A
Day0
Day3

Figure 6

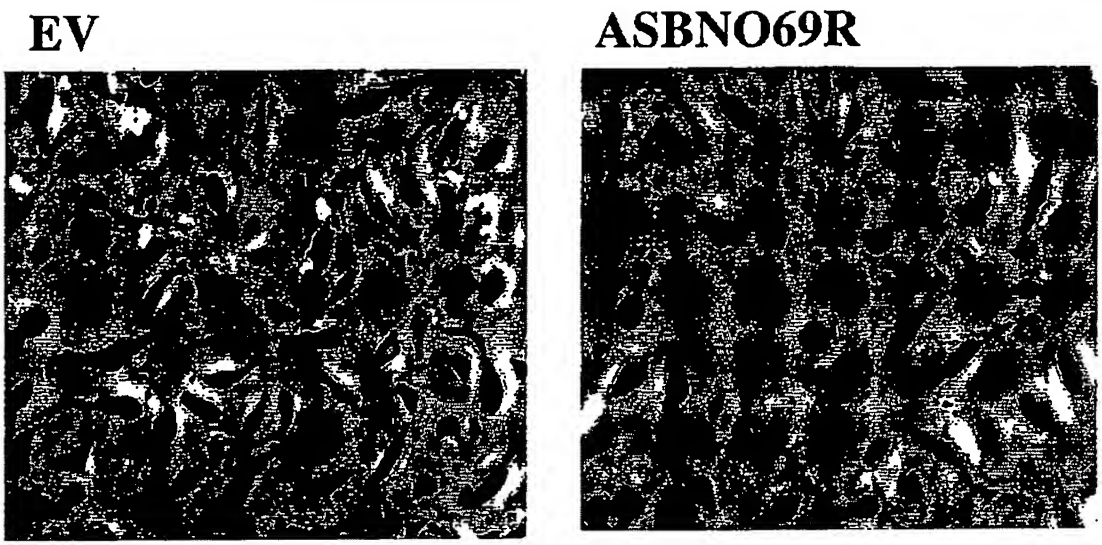


Figure 7

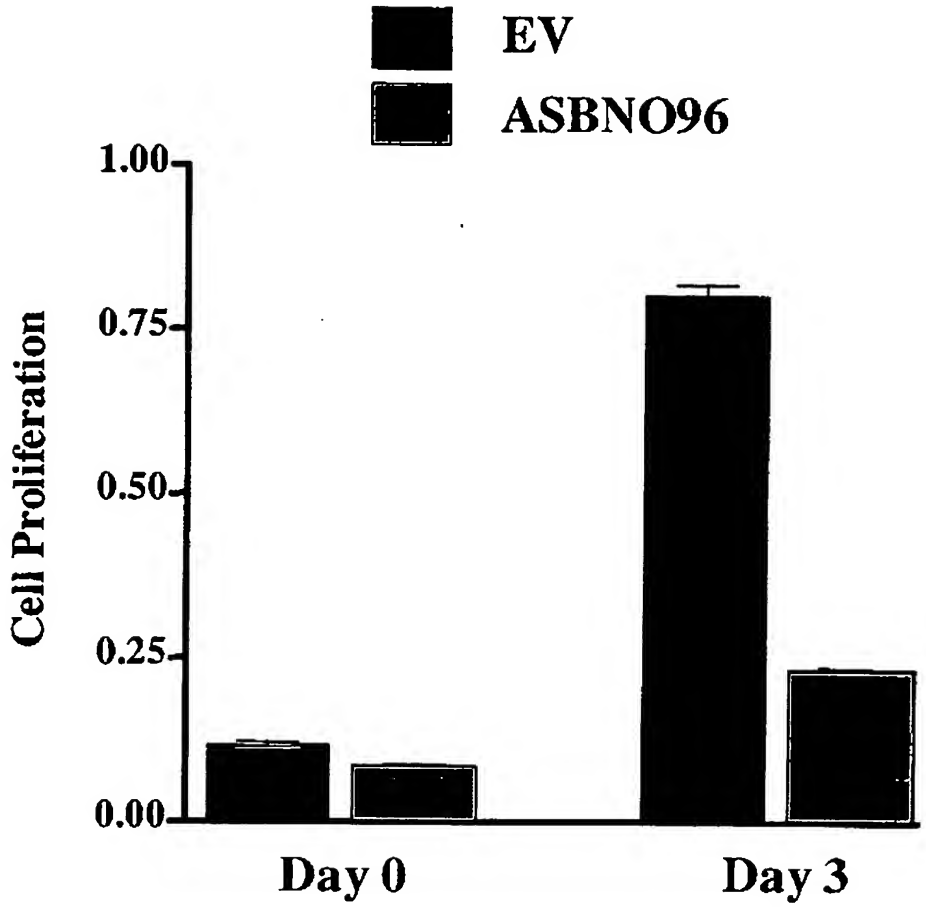


Figure 8

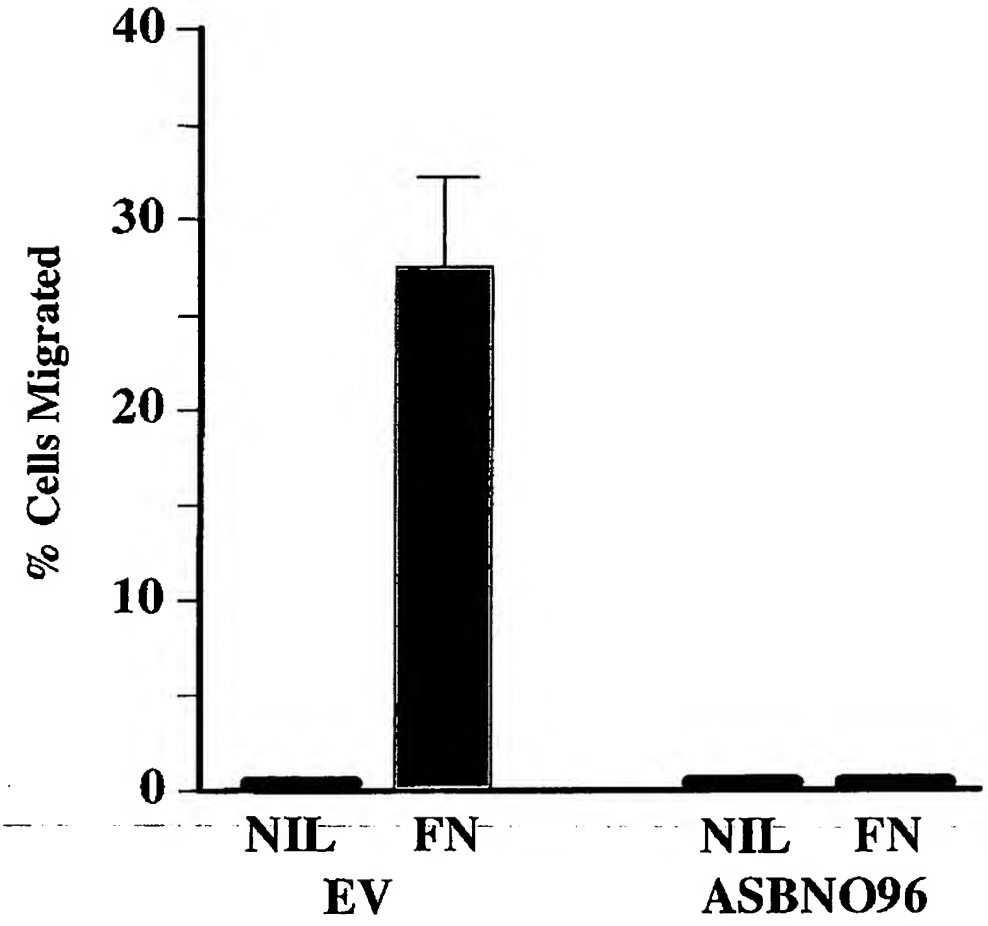


Figure 9

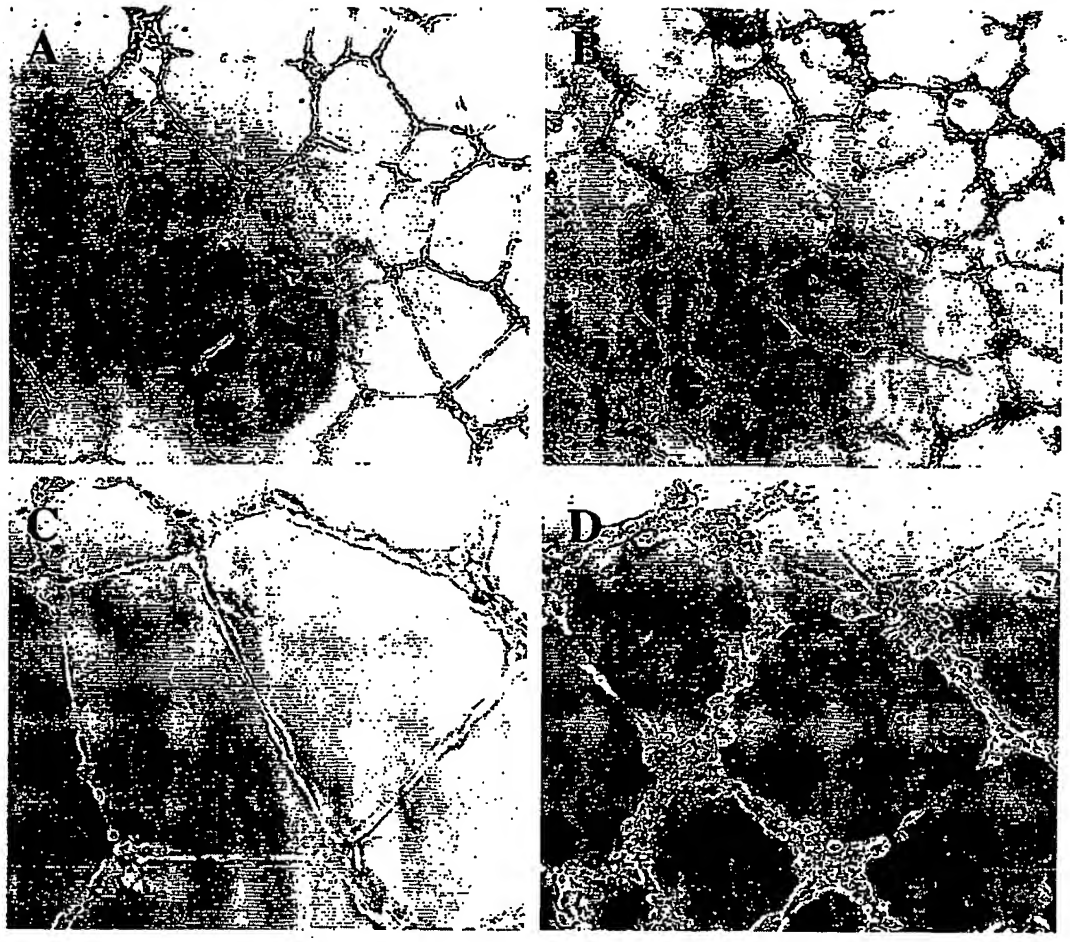
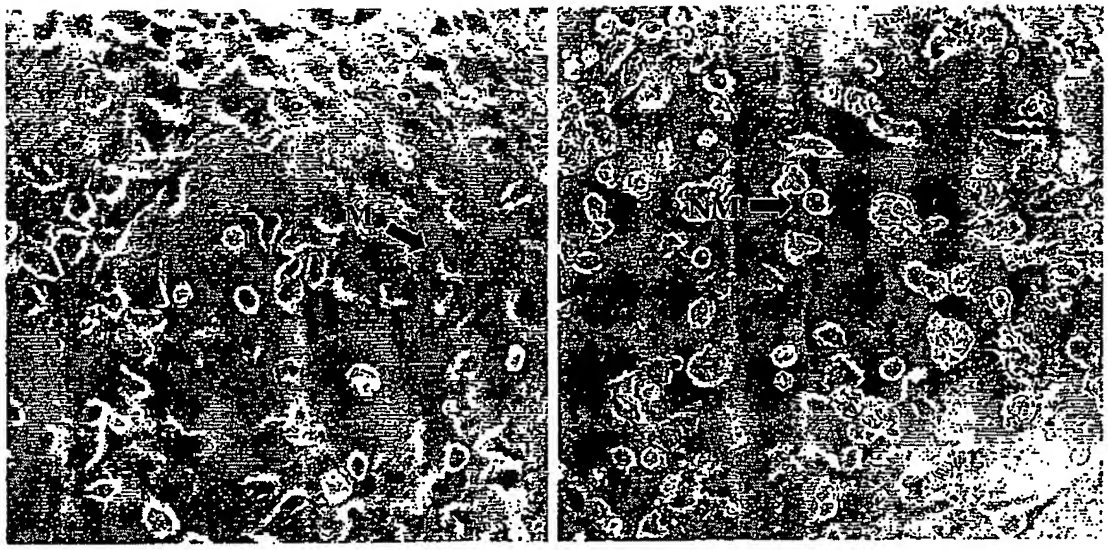


Figure 10



EV ASBNO96

Figure 11

	Basal E-selectin (MFI)	4-hr TNF Induced E-selectin (MFI)
Normal HUVEC	0.03	26.5
EV	0.6	20.9
ASGNG12	0.9	17.6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01282

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
SEE BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
SEE BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Swiss Prot: SEQ ID Nos 1-10
GenBank: SEQ ID Nos 1-10

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Genome Res 11, pp 422-435 (2001) Wiemann et al "Towards a catalog of human genes and proteins: sequencing and analysis of 500 novel complete protein coding human cDNAs"; and EMBL Accession No AL136646; 20.03.2002; relevant to SEQ ID No 1	1-10 and 24
X	EMBL Accession Number AL031428 "Human DNA sequence from clone RP-1-184J9 on chromosome 1p35.1-36.12". 07.02.2000; relevant to SEQ ID No 2	1-10 and 24
X	Genome Res 8, pp 1097-1108 (1998) "Toward a complete human genome sequence"; and GenBank Accession No AC004892; 21.12.1999; relevant to SEQ ID Nos 3, 5 and 9	1-10 and 24

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
31 October 2002

Date of mailing of the international search report
08 NOV 2002

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE
PO BOX 200, WODEN ACT 2606, AUSTRALIA
E-mail address: pct@ipaustralia.gov.au
Facsimile No. (02) 6285 3929

Authorized officer

MADHU K. JOGIA
Telephone No: (02) 6283 2512

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01282

C (Continuation). **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL Accession Number AL049796 "Human DNA sequence from clone RP4-561L24 on chromosome 1p22-31.1". 25.05.2000; relevant to SEQ ID No 4	1-10 and 24
P,X	Gene 293, pp 47-57 (2002) Rupp et al "Identification, genetic organization and mRNA expression of *CRELD1*, the founding member of a unique family of matricellular proteins"; and GenBank AF 452623; 05.07.2002; relevant to SEQ ID No 6	1-10 and 24
P,X	GenBank Accession No AK056493. "NEDO human cDNA sequencing project". 01.08.2002; relevant to SEQ ID No 7	1-10 and 24
P,X	GenBank Accession No AC097639 "Homo sapiens chromosome 3 clone RP11-384L8, complete sequence". 30.04.2002; relevant to SEQ ID No 8	1-10 and 24
P,X	GenBank Accession No AC098482. " Homo sapiens chromosome 3 clone RP11-839L21, complete sequence". 18.05.2002; relevant to SEQ ID no 10	1-10 and 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01282

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

SEQ ID Nos 1-114 constitute 11 different inventions, according to current practice. The applicant on invitation has identified as the first invention SEQ ID Nos 1-10.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:Related to SEQ ID Nos 1-10; see Supplemental Box

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: II

The international application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept. The fundamental test for unity of invention is specified in Rule 13.2 of the Regulations under the PCT.

"Where a group of inventions is claimed in one and the same international application, the requirement of unity of invention referred in Rule 13.1 shall be fulfilled only where there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical feature" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, make over the prior art."

The problem addressed by the present application is the identification of genes to be differentially expressed during the angiogenesis process. The solution provided by the claims resides in the use of 114 polynucleotide sequences and 101 polypeptide sequences. These sequences fall within a vast number of groups (based on at least 60 different gene type groups) as illustrated in Tables 1 and 2 of the present application.

The general concept underlying the present application appears to reside in the nucleic acid molecules encoding a polypeptide that has a role in angiogenesis. However, these molecules can only be considered to constitute a special technical feature if these molecules, considered as a single group, make a substantial contribution over the prior art. There is no indication in the present application that the isolation of the molecules encoding the proteins or the protein per se is inventive.

The following selection of documents disclose the study of genes and proteins displaying properties associated with angiogenesis:

J Cell Biochem 53(4), pp 360-72 (1993) Mallery et al "Modulation of human microvascular endothelial cell bioenergetic status and glutathione levels during proliferative and differentiated growth"

Int J Cancer 93 (4), pp 497-506 (Aug 2001) Rozic et al "Cyclooxygenase inhibitors retard murine mammary tumor progression by reducing tumor cell migration, invasiveness and angiogenesis"

Gen Pharmacol 35(5), pp 277-85 (2000) Wahl et al "Effects of microenvironmental extracellular pH and extracellular matrix proteins on angiostatin's activity and on intracellular pH.

These documents disclose genes which are differentially expressed during angiogenesis (Mallery et al), models for determining proteins linked with such processes (Rozic et al) and that angiogenic agents target migratory and proliferative cells (EC) in the process of forming new vessels, resulting in growth inhibition or cell death. While the present invention appears to rely on screening of genes for angiogenesis activity, these techniques are well known in the art and cannot be considered to be an inventive concept. Furthermore, the present invention does not provide any advantage, produce any unexpected result or overcome any difficulty associated with the use of these techniques.

..ctd..

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: II

Since there is no obvious special technical feature, it is appropriate to use the Markush approach to analyse the present set of claims. Claims 1, 2, 11, 15 and 16 in particular define alternatives, in a so-called Markush style of drafting. The application of the test of Markush claims gives the following results:

(A) the common property is molecules encoding proteins or claims to proteins, per se;

(B) (1) no common structure is evident from the disclosure of sequences 1-114;

(B) (2) there is no single recognised class of compounds embracing all of the molecules, as the molecules appear to belong to different classes eg, see Tables 1 and 2, each carrying out different biological functions.

It is contrary to normal classification to group together such different molecules, consisting of a vast number of groups resulting in a vast number of different inventions.

As a service to the applicant, multiple inventions, as specified by the applicant, will be searched for a single search fee with the proviso that the total number of nucleic acid sequences or proteins associated with the combination of inventions is no greater than 10. Examples of such combinations which would be acceptable as these contain no more than 10 molecules would be (with reference to Tables 1 and 2 of the present application):

SEQ ID Nos 1-10, 11-20, 21-30, etc

This offer is independent of unity consideration and is provided solely as a service to the applicant. Included in any such search fee would be the polypeptide encoded by the nucleic acid molecules. All of the 114 nucleic acid sequences would be searched for the payment of 10 additional search fees.

For the initial search fee the following inventions will be searched:

SEQ ID Nos 1-10.